EFFECT OF HUMAN MALE PATIENT DIAGNOSIS ON SYNGAMY TIMING AND BLASTULATION USING TIME-LAPSE TECHNOLOGY

By

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BSc Human Physiology, Psychology & Genetics

BSc (Hons) Genetics

Thesis presented in partial fulfilment of

the requirements for the degree of

Master of Science (Reproductive Biology)

In the Faculty of Medicine and Health Sciences

at Stellenbosch University

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April 2019

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SUMMARY

BACKGROUND: Improving embryo selection for embryo transfer is essential in Assisted Reproductive Technologies (ART), especially in single embryo transfer settings to reduce multiple birth rate. Current selection methods are largely based on assessment of morphological characteristics. Morphological blastocyst parameters have been shown in literature to correlate with implantation and live birth success rates, while female age is well established to affect overall assisted reproductive technology outcomes. More recently, kinetic parameters from time lapse (TL) technology have also emerged as additional selection characteristics during embryo development. As of yet, the effect of certain patient factors on kinetic parameters is to be elucidated. Syngamy is the merging event of maternal and paternal deoxyribonucleic acid (DNA) during fertilization and can be accurately determined / visualized using TL. The functional centrosome within the spermatozoa contributes to chromosome alignment and combination, therefore possibly impacting syngamy. The paternal genome from the spermatozoon also possibly contributes to blastulation on day 5 of embryo development by playing a role in the embryo genome activation, which drives advanced embryo development. The kinetic time points generated by TL technology during embryo development, can be used to examine the potential effect of male fertility diagnosis on syngamy and blastulation. This may elucidate whether syngamy timing is a relevant kinetic parameter that can allow more accurate selection and prediction of good quality embryos.

AIMS: Primary aim: To investigate the possible measurable direct effect of male fertility prognosis using TL, on the time duration to the syngamy fertilization event.

Secondary aim: To investigate the subsequent effect of male fertility prognosis using TL, on syngamy timing and the resulting advanced embryo development and blastocyst quality.

MATERIALS AND METHODS: This was a retrospective study that was conducted from 2017 to 2018 at Wijnland Fertility clinic on de-identified, aggregated TL patient embryo data from 2013 to 2016. Data was filtered according to inclusion and exclusion criteria and categorized according to defined male prognosis groups (good prognosis (GP), poor prognosis (PP), and very poor prognosis (VP)). Data was submitted for statistical analysis (statistical significance: $P < 0.05$). Female age was compensated for during statistical analysis.

RESULTS: Results indicated that all three male prognosis groups (GP, PP, VP) had similar rates of normal fertilization (60.12%, 58.84%, and 54.29%, $P = 0.19$) and subsequent blastulation (64.75%, 69.61%, and 63.28%, $P = 0.25$). The GP group showed significantly shorter syngamy timing compared to the VP group (19.73 ± 3.69 hours vs 20.80 ± 4.71 hours, $P = 0.02$). Shorter syngamy timing significantly correlated with increased blastocyst expansion on day 5 of embryo development in all groups ($P < 0.01$). A shorter syngamy timing significantly correlated with A-grade trophectoderm epithelium (TE) quality compared to B-grade ($P = 0.02$). A-grade TE in the GP group ($P = 0.05$) and VP group showed significantly shorter syngamy timing compared to B-grade in the VP group ($P = 0.04$). Syngamy timing did not show any significant correlation with inner cell mass (ICM) quality ($P = 0.36$) or between male prognosis groups ($P = 0.13$).

CONCLUSION: Results indicated that although fertilization and blastulation rate amongst the male prognosis groups were similar, poorer male prognosis may lead to extended syngamy timing and poorer blastocysts, impacting expansion and trophectoderm epithelium quality specifically. Since these two blastocyst parameters are the most important indicators of embryo implantation potential, syngamy can therefore be an early predictive marker for blastocyst quality, irrespective of initial sperm prognosis.

OPSOMMING

AGTERGROND: In die veld van reproduktiewe biologie en *in vitro* bevrugtingmetodes is enkel embrioterugplasing belangrik vir die vermindering van meervoudige geboortesyfers. Dit is dus essensiëel om embrioseleksie te optimeer. Huidige seleksiemetodes is grootliks gebasseer op morfologiese evaluering. Kinetiese parameters is onlangs ontwikkel, met behulp van tydsverloop-tegnologie (TL), wat addisionele embrioseleksie hulpmiddels bied. Die effek van spesifieke pasiëntfaktore op kinetiese parameters is tans nog eksperimenteel en nie ten volle ondersoek, bewys of beskryf nie. Vroulike ouderdom is wel reeds bewys as een van die hoof rolspelers in algehele reproduktiewe *in vitro* behandelingsuitkomste. Singamie is die samesmelting van vroulike en manlike deoksiribonukleïensuur (DNS) tydens die proses van bevrugting en kan duidelik gevisualiseer word met TL. Die funksionele sentrosoom binne die spermatosoön is verantwoordelik vir chromosoom belyning en samesmelting. Spermatosoa speel dus ‘n belangrike rol tydens die proses van singamie. Die manlike genoom, afkomstig van die spermatosoön, dra by tot blastosering op dag 5 van embrio-ontwikkeling en die aktivering van die embriogenoom gedurende gevorderde embrio-ontwikkeling. Kinetiese tydspunte gedurende embrio-ontwikkeling, met behulp van TL, kan gebruik word om die potensiële effek van manlike fertiliteitsdiagnose op singamie en blastosering te ondersoek. Bevindinge kan moontlik lei tot meer akkurate evaluering van embriokwaliteit en dus meer akkurate embrioseleksie vir embrioterugplasing.

DOELWITTE: Primêre doelwit: Ondersoek die moontlike meetbare effek van manlike fertilliteitsprognose op die tydsverloop tot singamie, waartydens die spermatosoön noodsaaklike sentrosome voorsien.

Sekondêre doelwit: Ondersoek die effek van manlike fertiliteitsprognose op die gevolglike gevorderde embrio-ontwikkeling, waartydens die manlike genoom ‘n aktiewe rol speel in blastosistontwikkeling en kwaliteit.

MATERIALE EN METODES: Hierdie was ‘n retrospektiewe studie vanaf 2017 tot 2018 by Wijnland Fertiliteitskliniek op ‘n onïdentifiseerbare, saamgevoegde TL databasis van *in vitro* bevrugtingsgevalle se embrio-ontwikkeling op data vanaf 2013 tot 2016. Data was volgens die insluit- en uitsluitingskriteria gefiltreer en gekategoriseer volgens bepaalde manlike prognosegroepe (goeie prognose (GP); swak prognose (PP); baie swak prognose (VP)). Die data was daarna ingehandig vir statistiese analise (statistiese beduidenheid: $P < 0.05$). Die moontlike verwagte effek van vroulike ouderdom was in berekening gebring tydens statistiese ontleding.

RESULTATE: Resultate dui aan dat al drie manlike prognosegroepe (GP, PP, VP) soortgelyke normale bevrugting- (60.12%, 58.84%, en 54.29%, $P = 0.19$) en blastoseringwaardes getoon het (64.75%, 69.61%, en 63.28%, $P = 0.25$). Die GP groep het ‘n korter singamie tyd getoon in vergelyking met die VP groep (19.73 ± 3.69 ure teenoor 20.80 ± 4.71 ure, $P = 0.02$). Korter tydsverloop tot singamie in alle groepe het beduidend gekorreleer met verder gevorderde blastosistvergroting op dag 5 van embrio-ontwikkeling ($P < 0.01$). Korter tydsverloop tot singamie het ook gekorreleer met trofektoderm kwaliteitsgradering, spesifiek tussen A- en B-graad trofektoderm ($P = 0.02$). Tydsverloop tot singamie was beduidend korter vir A-graad trofektoderm in die GP groep ($P = 0.05$) en VP groep in vergelyking met B-graad trofektoderm in die VP ($P = 0.04$). Tydsverloop tot singamie het geen beduidende korrelasie getoon met binneselmassa kwaliteit oorhoofs ($P = 0.36$), asook nie tussen die manlike prognosegroepe ($P = 0.13$) nie.

GEVOLGTREKKING: Resultate dui daarop dat alhoewel bevrugting- en blastoseringwaardes van die manlike prognosegroepe soortgelyk was, het swakker manlike prognose gelei tot verlengde tydsverloop tot singamie en gevolglik ook tot swakker blastosist kwaliteit. Die effek van verlengde singamie was veral op blastosist vergroting en trofektoderm kwaliteit waargeneem. Aangesien singamie spesifiek met hierdie twee belangrike blastosist parameters korreleer, wat goeie indikators van implantasie potensiaal is, kan singamie moontlik ’n vroeë indikator wees van blastosist kwaliteit, ongeag die sperm prognose.

DEDICATION

Dedicated to my parents,

Braam & Michelle Steyn

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude towards the following people and thank the institutions who contributed to this project:

- A special thank you to Dr. Marie-Lena De Beer, my research project supervisor, for her professional guidance, constructive recommendations, advice and assistance in keeping my progress on schedule. As well as her valuable support during my internship. (Chief Specialist Medical Scientist - Tygerberg Fertility Clinic, Dept. Obstetrics and Gynaecology, University of Stellenbosch, Tygerberg Hospital, Tygerberg).
- My co-supervisor, Mrs. Lydia Els-Smit, for her patience, guidance, enthusiastic encouragement and support, as well as her useful critique of this research project. I have learnt so much from her, not only as an embryologist, but also as a person! (Laboratory Director at Wijnland Fertility Clinic, Stellenbosch).
- Thank you to Prof. Martin Kidd for doing the statistical data analysis and his help and guidance on how to represent the data correctly. (Centre of Statistical Consultation, Dept. Statistical and Actuarial Sciences University of Stellenbosch).
- Mrs. Evelyn Erasmus for assisting me with the translation of the summary.
- Dr. Gerhard Hanekom for his suggestions and contribution to my thesis.
- Prof. Roelof Menkveld for assisting me with relevant articles.
- A special thanks to Dr. Johannes van Waart and Mrs. Lizanne van Waart, at Wijnland Fertility Clinic who allowed me to make use of their resources and data to conduct my study.
- I would like to thank my parents, Michelle and Braam Steyn, for always believing in me and for all their encouragement and support throughout my studies and personal life. They truly are one of the main reasons for all my achievements.
- To my brother and sister, Duan and Lanique Steyn, thank you for all your love and support!
- To Willie De Beer, thank you for all your love, support and encouragement during this time.
- To my grandparents, Jean and Alfonso Bredenkamp, thank you for always keeping me in your prayers and all your words of encouragement and support.

- Thanks to all the laboratory staff at Aevitas Fertility Clinic, Greg Tinney-Crook, Nicole Lans, Emma Ashley and Cherree Thwaits, for your support and advice.
- I would also like to thank the staff at Wijnland Fertility for welcoming me at the clinic during my internship to observe their daily operations and to learn from them.
- Thanks to my colleagues, Aqeel Morris, Libby Meiring, Camilla Janke and Dylan Ramsay for your support these past two years.
- All the glory to my Heavenly Father and Saviour, Jesus Christ. I would not have achieved what I have without Him blessing me with the ability to do so. I give Him all the honour for this thesis

ABBREVIATION LIST

General Assisted Reproductive Technology terms

ANOVA	Analysis of variance.
ART	Assisted Reproductive Techniques.
BQ	Blastocyst Quality.
CM	Compact Morula.
CPR	Clinical pregnancy rate.
DC	Distal centriole.
DFI	Deoxyribonucleic acid fraction index.
EB	Early Blastocyst.
EGA	Embryo genome activation.
GEE	Generalized estimating equations.
GP	Good prognosis.
GV	Germinal Vesicle (Immature Oocyte).
HA	Hyaluronic acid.
HDS	Highly deoxyribonucleic acid stainable.
ICM	Inner Cell Mass.
ICSI	Intracytoplasmic sperm injection.
IMSI	Intra cytoplasmic morphologically selected sperm injection.
IR	Implantation rate.
IUI	Intra-uterine insemination.
IVF	In vitro Fertilization.
LBR	Live birth rate.
LSD	Least significant difference.
MII	Metaphase II (Mature Oocyte).
MI	Metaphase I (Immature Oocyte).
OR	Ongoing pregnancy rate.
PB	Polar Body.
PC	Proximal centriole.
PCM	Pericentriolar material.
PCOS/PCO	Polycystic ovarian syndrome / Polycystic ovaries.
PGT-A	Preimplantation genetic testing for aneuploidies.
PGT	Preimplantation genetic testing.
PICSI	Physiological intracytoplasmic sperm injection.

PN	Pronuclei (2PN = normal fertilization, 0,1,3,>4 PN = abnormal fertilization).
PP	Poor prognosis.
PVS	Perivitelline space.
RNA	Ribonucleic acid.
SET	Single Embryo Transfer.
SCSA	Sperm chromatin structure assay.
SOP	Standard Operating Procedures.
TE	Trophectoderm Epithelium.
TL	Time-Lapse.
TUNEL	Terminal deoxynucleotidyl transferase-mediated deoxy-uridine triphosphate nick end labelling.
VP	Very poor prognosis.
ZP	Zona Pellucida.

Embryo developmental time-points

t0	Time of insemination.
tPB2	Time to second polar body extrusion.
tPNa	Time to pronuclear appearance.
tPNf	Time of pronuclear fading.
t2 -t8	Time of cleavages at specific cell stages (t2 = time to 2 cell division, t8 – time to 8 cell division).
tM	Time to morulation.
tSB	Time to start of blastulation.
tB	Time to full blastocyst.
tEB	Time to expanding blastocyst.
tHB	Time to hatching blastocyst.

Genetic terms

DNA	Deoxyribonucleic acid.
G_1	Stage of interphase in the cell cycle in which the cell grows and develops.
G_0	Non-dividing phase of the cell cycle (gap 0).
S-phase	Stage of interphase in the cell cycle where DNA replicates.
G_2	Stage of interphase in the cell cycle that follows DNA replication. The cell prepares for division.
Meiosis I	First phase of meiosis. In meiosis I, chromosome number is reduced by half.
Meiosis II	Second phase of meiosis. Events in meiosis II are essentially the same as those in mitosis.
M-phase	Process by which the nucleus of a eukaryotic cell divides/cleaves.

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INTRODUCTION

Time-lapse (TL) technology emerged in the 1990's, with the first observations of fertilization in oocytes by Payne *et al.* (1997).

Multiple pregnancy outcomes are a concerning part of Assisted Reproductive Technology (ARTs) and single embryo transfer (SET) strategies are becoming the more preferred option to reduce resultant obstetric risks and poor neonatal outcomes. This necessitates the development of better embryo selection methods.

TL incubators allow embryologists to access kinetic data from embryo development, which could assist in improving selection methods. Embryo selection is usually based on embryo and/or blastocyst morphology. Many studies have been done using TL technology with the goal to improve embryo selection and predict assisted reproductive technology (ART) outcomes (Meseguer *et al.*, 2011, Basile *et al.*, 2014a, Basile *et al.*, 2014b, Rubio *et al.*, 2014, Wirka *et al.*, 2014, Goodman *et al.*, 2016, Chen *et al.*, 2017, Aparicio Ruiz *et al.*, 2018).

Syngamy is the merging of maternal and paternal deoxyribonucleic acid (DNA), which is one of the early fertilization events that can be annotated clearly and accurately. This time point has been included in many studies investigating ART outcomes, but not yet to investigate the effect of male fertility prognosis (Azzarello *et al.*, 2012, Ueda *et al.*, 2012, Aguilar *et al.*, 2014, Wirka *et al.*, 2014).

Male factor prognosis is categorized according to male diagnosis that is determined through standard semen analysis. The functional centrosome within the spermatozoa contributes to DNA alignment and combination, therefore possibly impacting syngamy. The paternal genome from the spermatozoon also plays a role in blastulation by contributing to the embryo genome, which drives advanced embryo development. However, syngamy timing and its interaction with male prognosis and subsequent blastocyst quality has not been investigated. Early embryo developmental events, such as syngamy could allow earlier prediction of blastocyst quality.

The main focus of this study is to investigate the effect of male fertility prognosis on syngamy timing and blastocyst quality by making use of time-lapse technology, which will be substantiated in the literature review.

In **Chapter 1** background information as mentioned above is reviewed and compiled as a **Literature Review.**

In **Part 1** of the Literature Review an overview and introduction of **ART** is given.

In **Part 2** the main technology used in this study, **TL** systems, is discussed.

In **Part 3** the determination of **male factor prognosis** is described and discussed.

In **Part 4** the **process of oogenesis** is broadly discussed, as well as **female factors** affecting fertility.

In **Part 5** the **activation** of the **oocyte** and the **fertilization** process are discussed.

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In **Part 9** the Literature Review is concluded and final remarks are stated.

In the remainder of **Chapter 1**, the research question, hypothesis, aims and objectives are stated.

In **Chapter 2** the Materials and Methods are detailed, which include:

Part 1 - The Study design, **Part 2** - Study population, **Part 3** - Exclusion criteria, **Part 4** - Sample size, **Part 5** - Division into male prognosis groups, **Part 6** – Data Management, **Part 7** - ART methods used, **Part 8** -Semen analysis and diagnosis determination, **Part 9** – Time-lapse incubation equipment, **Part 9.1** - Equipment details of the EmbryoScope^{TM} incubator, **Part 9.2** - Time-lapse embryo culture dish preparations **Part 10** - Embryo annotation and syngamy determination, **Part 11** - Embryo blastocyst evaluation, **Part 12** – Flow diagram of the data management, **Part 13** – Limitations of the study, **Part 14** -Ethical considerations and **Part 15** - Statistical analysis.

In **Chapter 3** the **Results** of the study are presented.

In **Chapter 4** the results are reviewed in the **Discussion** section.

In **Chapter 5** the **Conclusion** of the findings of the study is formulated.

In **Chapter 6** the **Future prospects** are discussed.

In **Chapter 7** the cited **References** throughout the thesis are alphabetically listed.

In **Chapter 8** the **Appendices A-G** is included.

CHAPTER 1

LITERATURE REVIEW

1. Overview of Assisted Reproductive Technologies (ART)

ART (Assisted Reproductive Technologies) have been developed to overcome certain infertility diagnoses, with the first human birth resulting from *in vitro* fertilization (IVF) treatment, dating back to 1978. IVF has been successful for the treatment of couples with female factor infertility, which include tubal disease, endometriosis and unexplained infertility (Devroey and Van Steirteghem, 2004). Palermo *et al.* (1992) published on the first pregnancies and deliveries that resulted from intracytoplasmic sperm injection (ICSI). ICSI was specifically intended to treat male factor infertility and is currently the technique of choice for *in vitro* treatment worldwide to improve fertilization and pregnancy success. This procedure entails the selection of one spermatozoon based on morphological appearance, to be injected into one oocyte by means of micromanipulation needles fitted to an inverted microscope.

IVF is usually indicated as treatment for patients who have a good male prognosis and normal diagnosis. IVF entails insemination of oocytes by adding processed spermatozoa to oocytes and allowing spermatozoa to penetrate and fertilize the oocyte in a more natural selecting *in vitro* fashion, which resembles *in vivo* circumstances as close as possible.

A study done by Plachot *et al.* (2002) evaluated these two insemination methods, conventional IVF and ICSI, in patients with either one or a combination of male factor infertility which included: moderate oligozoospermia, asthenozoospermia and/or teratozoospermia, as well as obstructive and non-obstructive azoospermia. Overall results showed no significant difference between ICSI and IVF in fertilization rate, embryo morphology or developmental, pregnancy and implantation rates.

Yoeli *et al.* (2008) conducted a study on sibling oocytes inseminated by IVF and ICSI methods to determine whether there is a difference in embryo quality. They found higher fertilization rates in ICSI than in IVF (67.1% vs 43.6%), but found no significant difference in the embryo quality. They postulated that embryo quality did not depend on insemination method, but rather on inherent gamete factors.

A meta-analysis done by Johnson *et al.* (2013) showed that ICSI compared to conventional IVF gave better fertilization rates and decreased risk of total fertilization failure in couples with unexplained infertility.

Embryo quality is an important factor for its successful implantation into the endometrium (Oron *et al.*, 2014). It is dependent on the physiological function of both male and female gametes to complete important developmental milestones during embryo development.

In the effort to improve ICSI outcomes, two variations thereof have been developed in ART treatment, namely physiological intra-cytoplasmic sperm injection (PICSI) and intra cytoplasmic morphologically selected sperm injection (IMSI). These variations aim to improve sperm selection based on maturity or morphology and consequent ART outcomes in patients who have either had failed or poor ICSI cycle outcomes. Poor outcomes may be due to fertilization failure, failed blastocyst formation, low-quality blastocyst yield or chromosomal aberrations (Mokánszki *et al.*, 2014, Luna *et al.*, 2015, Gatimel *et al.*, 2016, Orief *et al.*, 2016, Erberelli *et al.*, 2017).

PICSI is based on the principle of hyaluronic acid (HA) binding. HA is present around the oocyte as part of the cumulus oocyte complex (COC), which acts as a selector for mature spermatozoa, which have HA-receptors, to bind to the oocyte (Beck-Fruchter *et al.*, 2016). Spermatozoa that bind to HA, are possibly more mature with less DNA fragmentation and fewer chromosomal mutations. However, literature shows contradicting evidence to whether PICSI is advantageous in comparison to the conventional ICSI (Beck-Fruchter *et al.*, 2016, Erberelli *et al.*, 2017).

IMSI is based on high magnification (>6000 times) in order to better identify and select morphologically normal spermatozoa, which correlate with decreased DNA fragmentation (Orief *et al.*, 2016). However, literature also shows contradicting evidence regarding the improvement in ART outcomes when implementing IMSI in comparison to traditional ICSI (Luna *et al.*, 2015, Gatimel *et al.*, 2016, Orief *et al.*, 2016).

In further effort to maximize the chance of implantation, the transfer of multiple embryos, mostly 2 to 3 embryos, have been common practice. This has also led to high multiple pregnancy rates with increased risk for perinatal complications, which include low birth weight and premature delivery, as well as risks for the mother (Papageorghiou *et al.*, 2006, Vega *et al.*, 2016). It has been shown that there is an increase in morbidity and mortality from preterm births, which increase with multifetal pregnancies (Haas *et al.*, 2016). In order to reduce multiple pregnancies and the obstetric risks that are associated with them, more laboratories are favouring single embryo transfers (SET) (Ahlström *et al.*, 2011). Therefore, embryo selection has become a crucial task in IVF laboratory practice to identify the embryo most likely to result in a live birth.

2. Time-lapse (TL) technology for human embryo culture

Traditional embryo culturing and incubation are conducted in benchtop incubators, which allow embryologists to view and evaluate embryos daily at only one static time point. Embryos need to be removed from the incubator for evaluation, causing disruption of embryo culture conditions, which could distress the embryo if exposed to the external environment for too long (Kovacs, 2014).

TL imaging for the study of early embryonic development started in the 1990's (Campbell and Fishel, 2015). Payne *et al.* (1997) revolutionized the first time-lapse observations in human oocytes by recording the first steps in the process of fertilization. The recent emergence of commercial TL incubation technology has developed into a new era of morphokinetic information where standard embryo culturing, incubation and monitoring for evaluation can be achieved without disturbing culture conditions (Kovacs, 2014). TL incubators are installed with microscope-based cameras that are able to photograph embryos every few minutes and in combination with computer software, can create a video of every embryo's development. In some TL systems, additional embryo tracking software is included.

There are several patented TL systems available: the EmbryoScope^{TM} (FertiliTech, Denmark), the Primo Vision (Vitrolife, Sweden), the Eeva (Early Embryo Viability Assessment) (Auxogyn, United States), and the Miri (Esco, Denmark).

The EmbryoScope™ (FertilitTech, Denmark) is a non-humidified incubator with a built-in TL system and EmbryoViewer software to allow annotation of embryos. The camera system uses low intensity red (LED 635nm) illumination, able to visualize the embryo in 7 focal to 9 equidistal planes (Kovacs, 2014, Campbell and Fishel, 2015). This incubator can hold 6 EmbryoSlide® dishes, which can accommodate up to 12 embryos per slide per patient. The system can track up to 72 embryos in total from 6 patients. Imaging is performed by automatically moving the dish by means of a motorized holding tray to bring the embryos into camera view.

The Primo Vision (Vitrolife, Sweden) is a portable TL unit that is placed in a conventional incubator and connected to an external controlling device (Kovacs, 2014, Campbell and Fishel, 2015). This system uses a compact, inverted microscope with Hoffman contrast integrated optics and green (LED 550nm) illumination that is able to visualize the embryo in 11 focal planes. Dishes for this TL system can accommodate 9 or 16 embryos per patient, allowing group culture as well as individual embryo monitoring. One Primo Vision unit is required for each patient, and a complete system of six units can track up to 96 embryos from six patients. Imaging is performed statically, without moving the dish, therefore minimizing disturbances.

The Eeva (Early Embryo Viability Assessment) (Auxogyn, United States) system is also a TL unit that is placed in a conventional incubator. The imaging system uses a dark-field illumination in order to increase cell membrane definition and visualizes embryos in a single focal plane (Kovacs, 2014, Campbell and Fishel, 2015). The Auxogyn software allows the Eeva system to predict every embryo's potential of developing to blastocyst stage, by tracking blastomere cleavages and using evaluation markers in the first two days post-fertilization. The Eeva dish includes individual wells for up to 9 embryos and also allows group-culturing.

The Miri® TL (Esco, Denmark) system is an incubator with 6 separate chambers that can accommodate 14 embryos per CultureCoins dish and 84 embryos in total (Campbell and Fishel, 2015). The imagining system uses red LED illumination and has a built-in high definition (HD) resolution interface touchscreen. This systems' software has an assist function which automatically detects the first cleavages to compare actual timings to reference timings for easier embryo selection.

The choice of TL incubation system is usually based on each laboratory's requirements, budget and available space.

It is clear that TL imaging systems allow the embryologist to observe embryo development in greater detail with better insight into timing of events such as pronuclear formation, syngamy, cleavage events, cell cycle events, synchronicity of cell division and blastulation. TL allows laboratories to conduct continuous monitoring of the embryo's development and ensures that timing of embryo development events can be determined more accurately (Desai *et al.*, 2014). The images and data can be recalled at any stage, can be re-evaluated if needed and be exported for research purposes. Many studies have been conducted using TL systems to improve embryo selection and interesting results have been published (Rubio *et al.*, 2014, Goodman *et al.*, 2016, Chen *et al.*, 2017, Aparicio Ruiz *et al.*, 2018).

Goodman *et al.* (2016) conducted a prospective, randomized control study to determine whether morphokinetic data from the TL EmbryoScope™ system improved clinical pregnancy rate (CPR) and implantation rates (IR) compared to traditional dynamic evaluation. The study showed no improvement in CPR, but did show improvement in IR for embryos with better morphokinetic data. Aparicio Ruiz *et al.* (2018) completed a retrospective study to investigate the difference of embryo quality and euploidy rate in two TL incubator systems compared to a traditional benchtop incubator. Their results showed an increased good quality embryo rate in both TL systems compared to the benchtop incubator. One of the TL systems showed increased euploidy rates in comparison with another TL system, as well as compared to the conventional incubator.

Rubio *et al.* (2014) conducted a prospective, double blind and randomized control study to assess the reproductive outcomes between the EmbryoScope™ TL system, with its additional morphokinetic information and traditional incubators, based only on morphology. The main outcomes included IR, pregnancy, ongoing pregnancy rates (OR) and early pregnancy loss. Results showed that the EmbryoScope™ TL system had improved reproductive outcomes in comparison to standard embryo benchtop incubators.

A meta-analysis and systematic review of randomized controlled trials by Chen *et al.* (2017) showed there is not enough substantial evidence that TL is more effective or superior than conventional benchtop embryo incubation, but it is definitive that TL provides a large amount of data which provides an enhanced tool for research in the field of embryology that could lead to better embryo selection.

Key events that take place during embryo development can offer prediction of blastocyst development and assist in deselection of embryos with low probability of viability (Mölder *et al.*, 2015). Desai *et al.* (2014) conducted a study looking at time to syngamy, time to two cells, thee, four, five and eight cells, morula, start of blastulation and time to full blastulation by using an EmbryoScope^{TM} TL incubator. They found that the data generated from this embryo selection method can potentially enhance embryo selection and improve transfer outcomes. Wirka *et al.* (2014) did a study using time-lapse microscopy to identify atypical embryo phenotypes by looking at abnormal syngamy, abnormal first cytokinesis, abnormal cleavage and chaotic cleavage. They concluded that atypical phenotypes correlate to lower developmental potential of embryos compared to their control embryos (Wirka *et al.*, 2014).

Time lapse incubation of embryos from fertilization to blastocyst stage can thus generate morphokinetic data in a non-invasive setting of specific developmental events of every embryo, such as syngamy and blastulation. This provides valuable analysable information about embryo development to help improve embryo selection. Algorithms based on TL morphokinetic data have previously been proposed to improve embryo selection (Herrero and Meseguer, 2013).

A study by Wong *et al.* (2010) pioneered an algorithm based on time-lapse morphokinetic markers and associated gene expression profiles. They compiled a timeline from which they derived the algorithm to predict blastocyst formation in very early stages of embryo development, before the embryo genome activation (EGA). The findings reveal that the maternal messenger ribonucleic acids (mRNA's), originating from the oocyte, plays a role in the cleavage stages before EGA, but fertilization and spermatozoa factors have not been elucidated yet (Wong *et al.*, 2010).

In another study by Meseguer *et al.* (2011) in which they retrospectively analysed the morphokinetic embryo developmental parameters of 2903 oocytes and correlated this information with implantation. Results showed that non-implanting embryos had increased variance of morphokinetic parameters compared to implanting embryos. Their hierarchical tree which was formulated to compare morphology classes, showed that TL classification was more accurate. TL could therefore contribute to embryo selection and subsequently improve clinical outcomes in ART.

Basile *et al.* (2014a) studied the time between cleavages of euploid and aneuploid embryos, using the EmbryoScope^{TM} TL system, to contribute to algorithms to improve non-invasive selection of euploid embryos. Results showed that euploid embryos had different kinetic behaviour compared to aneuploid embryos, on which they based their proposed algorithm to aid in classification of probable euploid embryos.

Basile *et al.* (2014b) did a large multicentred retrospective study on morphokinetic markers and its ability to predict implantation potential of an embryo by developing an algorithm that can be applied in different IVF clinics. The results showed that morphokinetic data with regards to timing of cleavages to 3-cell and 5-cell, as well as timing of the second cell cycle correlated with implantation in clinics that used similar methods. The continuous development and improvement of such algorithms could assist in selecting better embryos with higher implantation potential.

Fishel *et al.* (2018) conducted a retrospective study to measure live birth outcome by ranking blastocysts according to morphokinetic-based algorithms. Their main time point evaluated was time to start of blastulation (tSB) until time of full blastulation (tB) and embryos were ranked from A to D. They used time to pronuclei fading (tPNf) as the reference time point (t0), where they found that IVF embryos had a longer tPNf of 1.95 hours post insemination compared to ICSI. The main conclusion was that TL morphokinetic based algorithms can rank embryos according to prediction of live birth, with greater discrimination abilities than traditional embryo morphology grading.

TL morphokinetic data could therefore be used to specifically investigate the impact that male spermatozoa diagnosis has on syngamy timing and advanced embryo development i.e. blastulation. Accurate syngamy timing, its correlation with male diagnosis and blastulation have not been studied and could contribute to future algorithm development.

3. Male factor prognosis

Male factor prognosis is determined through a standard semen analysis. Semen analysis outcomes are based on semen parameters, such as spermatozoa concentration, motility, and morphology. Multiple studies have shown that these parameters have a positive correlation with *in vitro* fertilization success and pregnancy (Coetzee *et al.*, 1998). Approximately 50% of IVF treatments are done because of male factor infertility (Nanassy and Carrell, 2008).

Semen analysis investigates parameters that determine the male factor prognosis. The World Health Organization (WHO, 2010) has set standard reference values for semen parameters and male diagnosis. Descriptions of each diagnosis can be found in Appendix A.

Semen parameters are determined by following protocols set out by the WHO manual, although different laboratories may follow modified references determined within their clinical setting. Spermatozoa concentration or count can be assessed by making use of a Neubauer haemocytometer, which is a counting chamber with gridlines that is covered by a special coverslip (WHO, 2010). The semen is diluted with sterile water and loaded onto the counting chamber, where spermatozoa are counted and a concentration is determined. The motility of the sample is determined on a wet preparation slide, which is a slide on which a drop of semen is placed and is covered with a coverslip to provide a chamber of approximately 20µm in depth (WHO, 2010). The percentage of motile spermatozoa is then estimated across different fields, of which at least 10 fields are examined.

Spermatozoa morphology is an important part of a semen analysis, which allows selection of spermatozoa that have the best potential to fertilize the oocyte (WHO, 2010). This is based on the findings of Menkveld and colleagues (Menkveld *et al.*, 1990), as well as other scientists, where normal spermatozoa classification was described from spermatozoa recovered from cervical mucus in the female reproductive tract and bound to the zona pellucida of the oocyte (WHO, 2010). Sperm morphology evaluation is performed by making smears of the semen sample and staining these in order to assess morphology, which should be based on 100 to 200 spermatozoa (Acosta and Kruger, 1996, Björndahl *et al.*, 2010, WHO, 2010). The percentage indicates the number of spermatozoa with a normal form.

Spermatozoa are classified into three different morphology categories, 1-4% (P-pattern), 5-14% (G-pattern) and >15% (N-pattern) (Björndahl *et al.*, 2010). Spermatozoa are graded by looking at multiple morphological factors: (1) head shape, (2) neck / midpiece, and the (3) principal and end piece, which is the tail (WHO, 2010). Borderline cases are assessed as abnormal (Acosta and Kruger, 1996, WHO, 2010).

The head is assessed according to its size, form and structure (Björndahl *et al.*, 2010, WHO, 2010). The size of the head should not be too large or too small, have an oval form, an acrosome of more than 30% and less than 60% of the head size, no head abnormalizes or duplications of structures (Björndahl *et al.*, 2010, WHO, 2010). The neck cannot be too thick or thin, cannot be bent and asymmetrical insertions of the tail is regarded as abnormal (Björndahl *et al.*, 2010, WHO, 2010). The tail should not be bent or coiled, duplicated, too short, or irregularly thick (Björndahl *et al.*, 2010, WHO, 2010). Cytoplasmic residues are an indication of spermatozoon immaturity and is considered abnormal (Björndahl *et al.*, 2010, WHO, 2010).

Since ICSI has been developed for treatment of poor male prognosis, spermatozoa with poor parameters (concentration, motility and morphology) can be used for fertilization, although poor prognosis in male factor infertility has shown to compromise clinical pregnancy outcomes after ART (Coetzee *et al.*, 1998). This is probably due to the fact that ICSI selects for the best possible sperm based on motility and morphology, but it does not necessarily select normal genetic and epigenetic spermatozoa profiles (Denomme *et al.*, 2018). Compromised genetics inherited from the spermatozoa most probably contribute to impaired embryo development after fertilization.

It has been shown that immotile or non-progressive spermatozoa have increased centriole abnormalities compared to motile spermatozoa (Sathananthan, 1994, Palermo *et al.*, 1997). Syngamy prevention in reversed vasectomized patients could be due to antibody formation against centrioles, and timing of pronuclei (PN) positioning could be affected when using compromised spermatozoa (Palermo *et al.*, 1997). Thus, poor male prognosis may affect the fertilization events.

In the literature, there is no consensus about the effect of male diagnosis and semen parameters on ART fertilization rate. Hourvitz *et al.* (1998) found a significantly lower fertilization rate of testicular retrieved spermatozoa, obtained from patients with either non-obstructive azoospermia or oligo-astheno-teratozoospermia (42%), compared to the control group of ejaculated spermatozoa (55.5%). Zheng *et al.* (2016) conducted a retrospective cohort study and found that patients with reduced motility in addition to severe oligozoospermia had a significantly decreased fertilization rate in comparison to only moderate oligozoospermia patients, as well as decreased rate of good quality day 3 embryos in comparison to the control group.

Verza Jr and Esteves (2008) found that patients with triple sperm defect diagnoses and patients with testicular spermatozoa from non-obstructive azoospermia diagnoses had lower fertilization rates in comparison to other spermatozoa diagnoses (63.4 ± 25.9%; 52.2% ± 29.3% and ~73% respectively).

Borges Jr *et al.* (2017) conducted a study to evaluate the effect of male factor infertility on ICSI outcomes in comparison to a tubal factor control group according to maternal ages. Their results showed that normal fertilization rates did not differ significantly between male factor (81.33 ± 19.92%) and tubal factor groups (84.31 ± 15.99%) in women older than 35 years of age. The only significant result was in cycle cancelation rate, which was higher in male factor patients. However, there were significant differences in women under 35 years of age, who had a higher fertilization rate in the tubal factor group (86.43 ± 14.08%) compared to the male factor group (82.66 ± 17.32%). They also reported a higher number of transferred embryos in the male factor group, but no differences were seen in the implantation rate and pregnancy rate between the groups. Their overall fertilization rate between the male factor group (82.28 ± 18.09%) and the tubal patency group (85.64 ± 14.81%) was also significantly different when female age groups were not taken into consideration. The blastulation rates (42.10± 26.64% vs 43.00 ± 29.0%) and embryo quality was not affected by male factor. Their final conclusion was that male factor infertility does not interfere with the success of ICSI and that ICSI overcomes the poor specific outcomes associated with male factor.

In contrast, results by Yerebasmaz *et al.* (2017) showed that patients receiving ICSI treatment due to male factor infertility and tubal-unexplained infertility had no significant difference in fertilization (50.8% vs 51.2%, respectively), implantation (35.1% vs 37.0%, respectively) or clinical pregnancy (26.2% vs 30.8%, respectively) rates. However, after investigating the method of acquiring the spermatozoa, either by surgical extraction or ejaculation, ejaculated spermatozoa had an increase in fertilization rate (43.5% vs 51.8%, respectively), although not statistically significant.

Bungum *et al.* (2004) aimed to determine the relationship between the status of the sperm chromatin structure assay (SCSA) and the outcomes of biochemical pregnancy, clinical pregnancy and delivery in IVF, ICSI and intrauterine insemination (IUI). SCSA results were measured as DNA fractionation index (DFI) and highly DNA stainable (HDS) cell fractions. Their results showed that the group with less than 27% DFI and less than 10% HDS had a significantly better chance of successful pregnancy and delivery. Results also showed that in patients with more than 27% DFI, ICSI had significantly better outcomes in comparison to IVF. They concluded that SCSA could predict the outcome of ART.

Weissman *et al.* (2008) did a case series of four couples with male factor infertility and failed IVF or ICSI cycles. These ICSI cycles used ejaculated spermatozoa, which resulted in poor embryo quality and repeated implantation failure. Standard practice only resorts to testicular retrieval of spermatozoa in azoospermic patients, but this study retrieved spermatozoa from the testis even though patients had motile spermatozoa in the ejaculate. In all four cases, the couples had positive implantation and ongoing pregnancy or delivery results. This might have been a small study, but suggests that testicular spermatozoa could be an alternative option for repeated IVF/ICSI failures. They speculated that these failures were due to spermatozoa DNA damage in the male reproductive tract.

Moskovtsev *et al.* (2010) compared DNA fragmentation of patients with failed oral antioxidant treatment in ejaculated spermatozoa to testicular spermatozoa. They performed terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) assays. The results showed that ejaculated spermatozoa had three times more DNA fragmentation (39.7 ± 14.8%) in comparison to testicular spermatozoa (13.3 ± 7.3%). The obvious conclusion was that patients who had failed oral antioxidant treatment, showed improved DNA quality from testicular retrieved samples in comparison to ejaculated samples.

Esteves *et al.* (2015) evaluated the ability of ICSI to overcome high sperm DNA fragmentation in male factor infertility. Male patients with oligozoospermia, as well as high DNA fragmentation, who had ICSI cycles using either ejaculated sperm samples or testicular sperm samples from surgical extraction or aspiration were included. They showed that testicular sperm had significantly lower DFI (8.3%) compared to ejaculated sperm (40.7%). The CPR between the testicular retrieved and ejaculated spermatozoa also differed with regards to CPR (51.9% vs 40.2%, respectively) and significantly differed in miscarriage rate (10.0% vs 34.3%, respectively) and live-birth rate (46.7% vs 26.4%, respectively). They concluded that testicular retrieval of spermatozoa in patients with oligozoospermia and high DNA fragmentation is an effective treatment option to improve ART outcomes.

In the field of ART, there is a wide variety of patients with different spermatozoa diagnoses. The possible effect which it could have on syngamy timing and blastulation rate is unknown. The few studies mentioned, suggest that there might be an influence of male patient diagnosis, which we will be investigating.

According to these studies, the quality of the spermatozoa affected fertilization and subsequent embryo potential and development. Therefore, the effects on early fertilization events, like syngamy timing and late embryonic development, such as blastulation is still unclear.

4. Oogenesis and female factors

Oogenesis is the process that produces female gametes. The ovaries are the site of oocyte production, where diploid primordial germ cells divide via mitosis to form oogonia. In the human female, oocytes form during fetal life, where each oogonium completes meiosis I, divides and arrests in meiosis at the prophase I stage, known as primary oocytes (Pierce, 2012, Coward and Wells, 2013). When puberty is reached, hormone levels stimulate the primary oocytes to complete meiosis I, where the oocyte proceeds to complete crossing over in prophase I, which allows intrachromosomal recombination to create new alleles on the chromatids (Pierce, 2012). Thereafter, metaphase I, anaphase I, telophase I and cytokinesis are completed (*see Figure 1.1*). The secondary oocyte and first polar body is formed and is distinguished by the proportion of cytoplasm allocated to each oocyte (Pierce, 2012). The first polar body extrusion characterizes a mature oocyte, it receives a small part of the cytoplasm and usually disintegrates. The secondary oocyte receives most of the cytoplasm, which is ovulated from the ovary and ready to be fertilized (Pierce, 2012, Campbell and Fishel, 2015). The second division, meiosis II, is delayed until contact with the spermatozoa, after the secondary oocyte is penetrated and the oocyte is activated (Pierce, 2012).

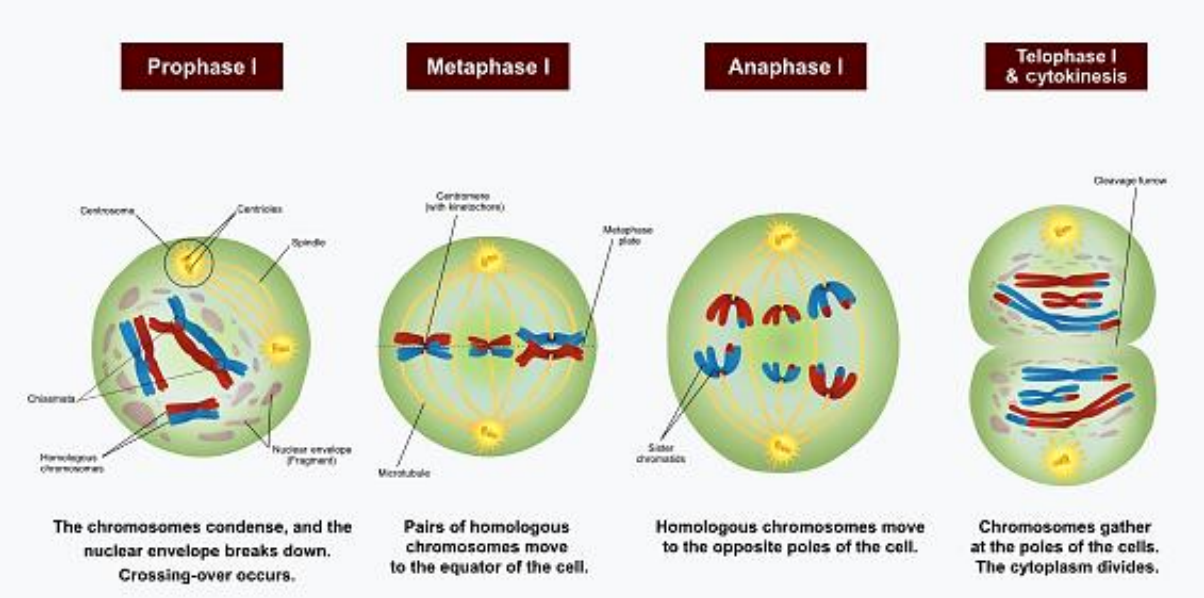

Figure 1.1 Diagram of the first meiotic phase.

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At birth, a female has approximately 1-2 million oocytes, which continuously declines as she approaches puberty (Liu *et al.*, 2011). At puberty only 300 000- 500 000 million oocytes remain and during the reproductive years women will ovulate 400-500 oocytes, with the remainder of the oocytes being lost through apoptosis (Liu *et al.*, 2011).

Female age is a confounding factor in reproduction, which also plays a role in the prognosis of ART. As women age, their fertility declines when oocyte quantity, the quality of the DNA and biological function decreases. Age is therefore a good predictor of ovarian reserve. It is clear that embryo aneuploidy increases and success rates in ART decrease with advancing female age (Lawler *et al.*, 2007, Liu *et al.*, 2011). Studies conducted in Canada and in the United States showed that success in ART treatment is significantly impacted by female age, with live birth rates (LBR) decreasing as maternal age increases (Liu *et al.*, 2011). This trend was generally seen in women in their mid-30's and onward, which continues until menopause. Some studies show that normal fertilization rate is not affected by advanced maternal age, although it does show an increase in abnormal fertilization rate (Grøndahl *et al.*, 2017).

Lawler *et al.* (2007) investigated the relationship between entry into syngamy and first cleavage as predictors of implantation on day 2 of embryo development, as well as the inter-relationship of female age. Pronuclear presence was evaluated 16 to 18hours post insemination and a second evaluation was done at 23 to 24 hours post insemination for ICSI and IVF. They concluded that there is a significant correlation between time of entry into syngamy and female age, with earlier syngamy associated with females under the age of 36 years (Lawler *et al.*, 2007). They also determined that implantation potential was higher for day 2 embryos when entry into syngamy occurred before 23 to 24 hours post insemination.

Apart from female age, female diagnoses are established by investigating tubal patency, ovulatory function, and standard fertility tests. Infertility is defined as the inability to conceive after a minimum of 12 months of frequent, unprotected intercourse during the ovulatory period of the female (Ray *et al.*, 2012, Gelbaya *et al.*, 2014, Abdelazim *et al.*, 2018).

Tubal patency was usually assessed through hysterosalpingogram and/or laparoscopy. The adhesion or obstruction of the Fallopian tubes could be due to infections, endometriosis or previous surgery. The prevalence of tubal factor infertility in sub-fertile groups is approximately 14% (Gelbaya *et al.*, 2014).

Endometriosis is characterized by endometrial-like tissue present outside the uterine cavity and its mechanism associated with female infertility is not completely understood (Dong *et al.*, 2013). A retrospective study by Dong *et al.* (2013) in patients with endometriosis who underwent IVF/ICSI treatment has shown that pregnancy outcomes were similar to patients with tubal factor diagnosis. Although there is contradicting literature that suggest that patients with endometriosis have poorer outcomes (Gelbaya *et al.*, 2014). Literature shows that 30% to 50% of infertile patients suffer from endometriosis (Dong *et al.*, 2013, Gelbaya *et al.*, 2014).

Normal ovulatory function is assessed by the evaluation of different ovulatory indicators such as, a rise in basal body temperature, changes in cervical mucus, serum luteinizing hormone surge or mid-luteal progesterone levels (Ray *et al.*, 2012). Polycystic ovarian syndrome (PCOS) is one of the causes of ovulatory dysfunction. The prevalence of PCOS is between 18% to 25% (Heijnen *et al.*, 2005, Gelbaya *et al.*, 2014). Heijnen *et al.* (2005) conducted a meta-analysis between PCOS and non-PCOS patients, in which it was concluded that patients in both these groups had similar pregnancy and LBR per cycle.

Idiopathic (also known as unexplained) infertility was diagnosed after all possible female causes are excluded and male semen analysis was normal (Ray *et al.*, 2012, Gelbaya *et al.*, 2014, Abdelazim *et al.*, 2018). According to the three studies cited, the prevalence of idiopathic fertility is between 8% and 40%. Unexplained fertility could be due to undetected problems in the reproductive process or lower fecundity of the couple. The prevalence of idiopathic infertility varies according to female age, inclusion criteria of the study population, as well as the investigations done before the diagnosis was made (Gelbaya *et al.*, 2014).

5. Fertilization and oocyte activation

Fertilization consists of a sequence of events that leads to the merging of a spermatozoon and oocyte, which entails the fusion of their haploid genomes and cytoplasmic contents in order to result in a diploid zygote (Klinovska *et al.*, 2014). The oocyte and the spermatozoon need to be mature for fertilization to be able to occur in the ampulla of the oviduct (Georgadaki *et al.*, 2016). Spermatozoa need to travel to the fallopian tubes after semen has been deposited in the vagina. This journey through the acidic environment of the vagina, passing through the cervical mucus, uterus and proceeding to the fallopian tubes, selects and eliminates many spermatozoa and results in only a few hundred spermatozoa reaching the oocyte (Anifandis *et al.*, 2014).

It is hypothesised that the cumulus mass of the oocyte secretes chemo-attractants that assist the spermatozoa to direct themselves towards the oocyte (Anifandis *et al.*, 2014). Spermatozoa need to penetrate the cumulus cells, as well as bind to and penetrate the zona pellucida (ZP) and plasma membrane, in order to successfully fertilize the oocyte. The penetration is only possible if the spermatozoa have undergone essential biochemical and biophysical changes. These changes are brought about by the process of capacitation and hyperactivation, also allowing the acrosome reaction to occur. This process involves the increase in pH and calcium ion (Ca^{+}) levels.

Capacitation is the activation of spermatozoa and is accompanied by hyperactivation, an elevated energy state which increases motility by vigorous flagellar movement (Georgadaki *et al.*, 2016); allowing detachment from the isthmus epithelium (Anifandis *et al.*, 2014). Capacitation involves the removal of cholesterols and other inhibitory factors such as sterols, and seminal plasma proteins (Anifandis *et al.*, 2014, Georgadaki *et al.*, 2016). Only spermatozoa that have completed capacitation have the ability to bind to and penetrate the ZP and fertilize the oocyte (Georgadaki *et al.*, 2016).

Oocyte activation involves several physical and chemical changes that occur in the oocyte. The first is an increase in ionic permeability of the plasma membrane (Elder and Dale, 2010), followed by the cortical reaction and ZP transformation, in order to prevent further penetration by other spermatozoa.

The extracellular matrix of the ZP comprises of three glycoproteins, ZP1, ZP2 and ZP3 (Georgadaki *et al.*, 2016) and the cumulus oophorous consists of granulosa cells that contain hyaluronan (Georgadaki *et al.*, 2016). The binding of the spermatozoon to the ZP3 protein triggers the acrosome reaction, which is an exocytosis reaction that releases enzymes such as hyaluronidase to disintegrate the cumulus oophorus (Anifandis *et al.*, 2014, Georgadaki *et al.*, 2016). Binding to the ZP activates mechanisms that increase Ca^{+} concentration and pH levels (Anifandis *et al.*, 2014). The acrosome membrane fuses with the plasma membrane of the spermatozoa, releasing peptidases and enzymes such as acrosine in order to penetrate the ZP (Anifandis *et al.*, 2014).

After the spermatozoa have bound to the ZP, and the acrosome reaction is initiated, acrosome-reacted spermatozoa are able to penetrate the ZP. This is due to the fact that the acrosome releases enzymes which also digest through the ZP, creating a pathway for the spermatozoa to enter the oocyte. These enzymes include acrosin, which is a protease, testicular serine protease 5 (TESP5) and a multi-subunit proteasome that has proteolytic characteristics (Anifandis *et al.*, 2014). The acrosome reacted spermatozoon which successfully penetrates the ZP and enters the perivitelline space is able to fuse with the oolemma of the oocyte (Klinovska *et al.*, 2014).

The cortical reaction occurs next in order to prevent polyspermy after one spermatozoon has successfully bound to the oocyte (Georgadaki *et al.*, 2016). This is achieved by modifying the ZP to be impenetrable to other spermatozoa. This process is initiated by the increase in Ca^{+} from the cortical smooth endoplasmic reticulum in a wave like manner, causing the cortical granules to rupture (Georgadaki *et al.*, 2016). The Ca^{+} increase activates the protein exchanger, which increases the pH by influx of Na^{+} and efflux of H^{+} (Georgadaki *et al.*, 2016). The release of the cortical granule contents leads to changes in the extracellular matrix, such as hardening of the vitelline envelope, and enzymes digest the ZP3 and ZP2 spermatozoa receptors to avoid any further binding of spermatozoa (Georgadaki *et al.*, 2016).

During gamete fusion, the membranes of the spermatozoon and oocyte fuse, which is believed to be dependent on the lipid membranes found on both gametes (Coward and Wells, 2013). Spermatozoa that have undergone the acrosome reaction are able to fuse due to the fact that essential factors on the membrane are exposed or altered by the process (Klinovska *et al.*, 2014). The equatorial region on the spermatozoon head is proposed to be the area where fusion is initiated (Klinovska *et al.*, 2014). The oocyte plasma membrane also appears to consist of a microvillar-free section that covers the meiotic spindle and microvillar-rich area, where fusion only occurs on the microvillar-rich part (Klinovska *et al.*, 2014). The fact that fusion occurs at specific regions on both gametes, might indicate that these regions contain a certain protein and lipid composition (Klinovska *et al.*, 2014). The fusion of the two gametes entail the merging of the oocyte's plasma membrane with the spermatozoon, as well as the combining of the cytoplasm's.

After gamete fusion, the oocyte needs to be activated in order to transcribe the necessary proteins and RNAs for fertilization and embryogenesis. Spermatozoa introduce the oocyte activating factor, which modifies the regulatory components for calcium homeostasis in the oocyte (Tesarik, 2005). Activation ensures that a mature oocyte is able to develop into a growing embryo. The mechanism by which activation is initiated is still unknown, but there are many models suggesting that it could be a calcium influx, membrane receptors or a soluble sperm factor model.

Meiosis is reinitiated and meiosis II is completed (*see Figure 1.2*): Prophase II, metaphase II, anaphase II, telophase II and final cytokinesis results in a haploid maternal genome and extrusion of the second polar body (Pierce, 2012). The maternal haploid genome is then able to combine with the paternal haploid genome. The haploid genomes of both gametes are visualized when the two pronuclei appear in the oocyte.

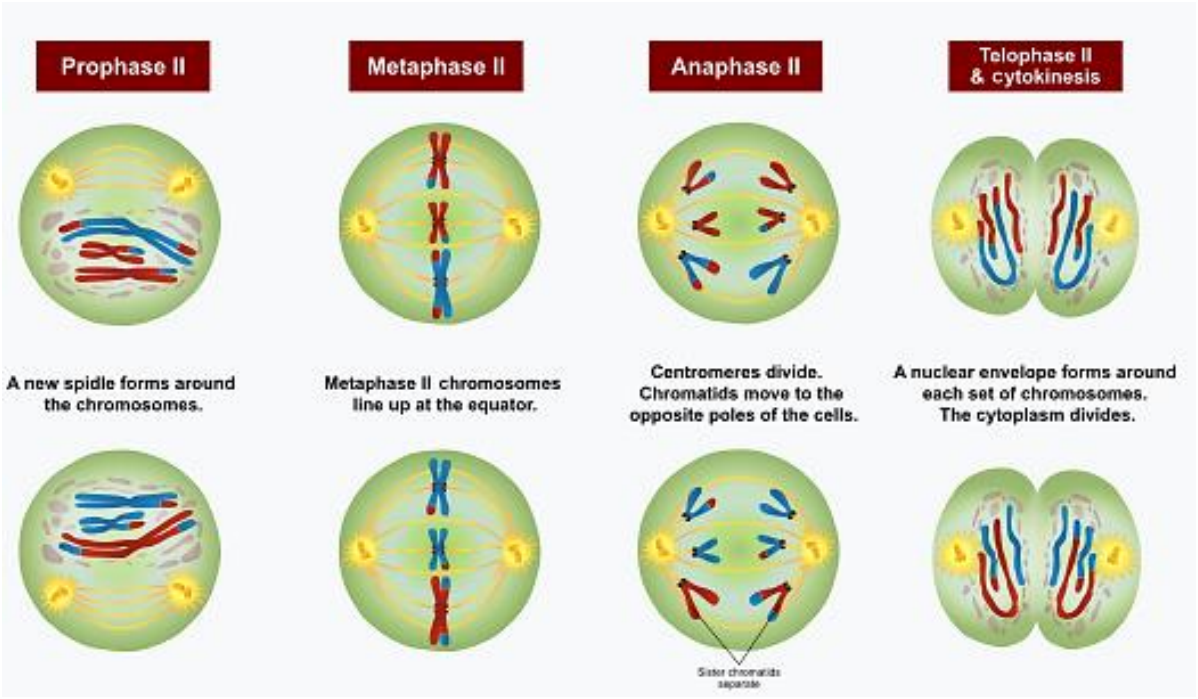

Figure 1.2. Diagram of the second meiotic phase.

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Maternal RNA and proteins can now be transcribed and translated; as well as the cytoskeleton rearranged in order to sustain embryo development and growth (Pierce, 2012). This process results in the eventual rise of a zygote (Pierce, 2012).

6. Pronuclei formation and pronuclear (PN) morphology

The extrusion of the second polar body is the first sign of fertilization, although this does not indicate normal fertilization (Campbell and Fishel, 2015). Normal fertilization of an oocyte is characterized by **pronuclear formation** and the presence of two pronuclei (PN) and two polar bodies (Balaban *et al.*, 2011). Studies have shown that post insemination timing of the second polar body extrusion was indicative of embryo implantation and morphology (Campbell and Fishel, 2015). The optimal time for second polar body extrusion and embryo implantation was investigated by Aguilar *et al.* (2014) in a TL study and shown to be between 3.3 and 10.6 hours post ICSI.

Nuclear membranes form around each of the decondensed male and female chromatin, creating two PN that contain the two sets of haploid chromosomes. The female pronucleus forms closer to the second polar body and appears simultaneously or slightly after the central formation of the male pronucleus (Campbell and Fishel, 2015). In the case of a zygote having one or three PN, laboratories generally annotate these as abnormal fertilizations.

The pronuclei need to be of equal size and in a central position; if they are not located centrally, it could be an indication of failed mechanisms within the fertilization process (Scott, 2003). The polar axis is formed after sperm entry and oocyte activation. At this stage, it is essential that the PN align correctly and that the polar axis forms for normal syngamy and first cleavage to take place (Scott, 2003). The pronuclear morphology has been used in order to predict arranged embryo development (Balaban *et al.*, 2011). Structures visible within the pronuclei, known as nucleolar precursor bodies (NPBs), have been used as embryo quality criteria based on their number and pattern display (Elder and Dale, 2010). However, it has been noticed with TL incubation and evaluation that these NPB patterns change and the arrangements are dynamic (Azzarello *et al.*, 2012).

In the cell cycle, mitosis cell division consists of two phases. The interphase, which consists of the G_1 (phase of cell growth), G_0 (Non-dividing phase), Synthesis-phase (DNA duplication stage) and G_2 (cell preparation for mitosis), and the M-phase which is the mitotic phase when cell division occurs (*see Figure 1.3*).

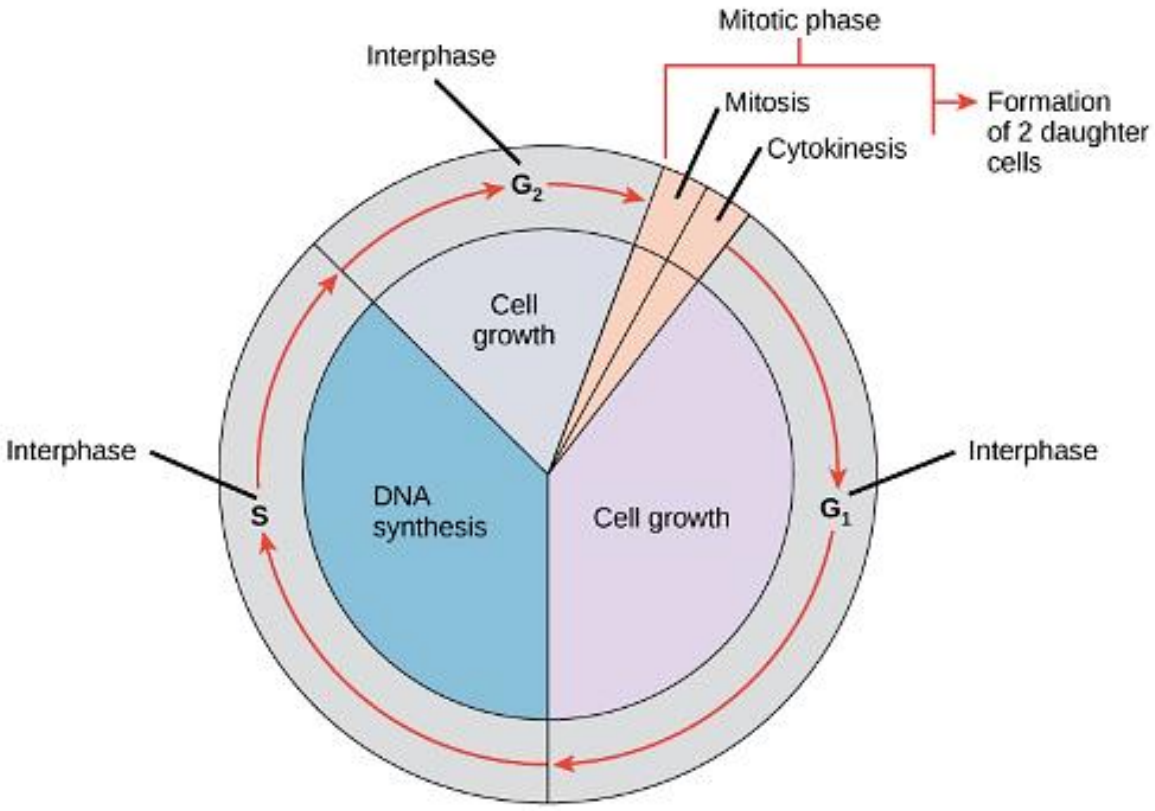

Figure 1.3. Diagram depicting the cell cycle consisting of the interphase and M phase. Image by: OpenStax College, Biology, https://cnx.org/contents/1tJ55Ot6@7/The-Cell-Cycle.

Studies using TL have shown that timing of pronuclear fading has a significant effect on embryo implantation, where a shorter timing of PN fading was linked to better implantation (Aguilar *et al.*, 2014). The length of the S-phase is determined from PN appearance until PN fading and Masai *et al.* (2010) reported a duration time for the S-phase of 7 to 8 hours. Aguilar *et al.* (2014) reported better implantation of embryos with a S-phase duration of 5.7 to 13.8 hours compared to embryos with a longer S-phase duration of >13.8 hours.

Hewitson *et al.* (2000) reviewed the possibility of the initiation of the S-phase differing between IVF and ICSI insemination methods, using rhesus monkey zygotes as a non-human model. They found that ICSI fertilized zygotes had abnormal nuclear remodelling which resulted in delayed onset of DNA synthesis. They concluded that ICSI possibly may result in chromatin damage when de-condensation of DNA occurs, accentuating the need for better pre-clinical assessment prior to use and acceptance of treatment.

The result of delayed onset of the S-phase, in the beforementioned study, was contradicted by Cruz *et al.* (2013) who showed that the differences in kinetic parameter time points between IVF and ICSI disappeared when pronuclei fading was used as the reference time point (t0) rather than time of insemination.

The fusing of the two pronuclei takes place during syngamy which is defined as the time of PN fading (Aguilar *et al.*, 2014). This takes place after the S-phase has been completed and before the M-phase initiates.

The S-phase is then preceded by the G_2 and M phase. Meseguer *et al.* (2011) reported a time duration of 4.04 hours from the time of PN fading until the time of first cell cleavage, which delineates the G_2 and M phases.

A few studies have investigated certain fertilization events such as pronuclei morphokinetics and syngamy timing and have indicated positive correlations with embryo quality. However, it is noted that studies differ in reference time-points and calculations used to investigate these events.

7. Syngamy and role of spermatozoa in the embryo genome

The fertilization process continues with the fusion of two haploid genomes, namely the maternal and paternal genomes to produce a diploid zygote (Coward and Wells, 2013), also known as **syngamy**. It has been proposed by Balaban *et al.* (2011) to evaluate syngamy at 23 +/- 1 hour post insemination.

After pronuclear formation, the pronuclei gradually migrate to the centre of the oocyte, until they are adjacent to each other. The pronuclear membranes disintegrate and the mitotic metaphase spindle forms. Between 18 and 24 hours after gamete fusion, the two sets of chromosomes come together in syngamy. Syngamy is described as the merging of maternal and paternal DNA, and can be visualized when the two pronuclei fade (Aguilar *et al.*, 2014).

The **mitotic spindle** is the structure that ensures the correct distribution of genomic material (Palermo *et al.*, 1997). The mitotic spindle is formed by the centrosome, which regulates the orientation of the microtubules to allow even dispersal of chromosomes (Palermo *et al.*, 1997).

Spermatozoa are the source of the **functional centrosome** after spermatozoa entry into the oocyte, which lacks centrioles (Palermo *et al.*, 1997). The function of the centrosome is to organize the microtubule network and nucleate the aster, which contains the microtubules (Palermo *et al.*, 1997). Previous research has proposed the theory that only one centriole is inherited by the zygote from the spermatozoa, but this has brought about confusion with regards to the number of centrioles provided by the spermatozoon (Schatten and Sun, 2009, Fishman *et al.*, 2018). The spermatozoon is able to provide two centrioles for each dividing daughter cell, leading to the unanswered question as to where the second centriole is coming from.

The spermatozoa's centriole-centrosome complex, comprises of a proximal centriole (PC) and distal centriole (DC). The PC and DC is located in the neck region of the spermatozoa where the basal plate and flagellum of the sperm join (Schatten and Sun, 2009, Fishman *et al.*, 2018). The dogma in literature was that the centrioles and surrounding pericentriolar material (PCM) was modified during spermatogenesis, called centrosome reduction. It was believed that the DC disintegrates, leaving only the functional PC.

However, new findings have confirmed a second atypical centriole, which is delivered to the zygote by the spermatozoa (Fishman *et al.*, 2018). Fishman *et al.* (2018) found that during spermatogenesis, the centrosome is remodelled as proteins are rearranged and enables spermatozoa to deliver two centrioles, the known typical centriole and the newly discovered, atypical centriole. This is achieved when proteins are redistributed amongst the PC, DC and PCM during spermatogenesis, which results in the DC not being disintegrated, but rather being remodelled into an alternative composition.

The spermatozoa thus provides two centrioles to the zygote, the typical centriole (PC), atypical centriole (DC) and the remodelled structural PCM (Fishman *et al.*, 2018). They found that the DC recruits the PCM *in vivo* to form the daughter centriole, providing two functional centrosomes which position at opposite spindle poles in the zygote. It is still unknown what the purpose of the remodelled structure is.

The spermatozoa play a role in the events that precede syngamy and directly affect syngamy itself. During male pronuclear formation, elongated microtubules originate from the spermatozoa centrosome (Van Blerkom, 1996). These microtubules come into contact with the female pronucleus to stabilize close proximity of the two PNs before syngamy occurs (Van Blerkom, 1996, Palermo *et al.*, 1997). The event of microtubule formation occurs after penetration but before DNA de-condensation and nuclear expansion (Van Blerkom, 1996). Figure 1.4 illustrates this process.

Figure 1.4. **a)** Illustration of the meiotic spindle during the first polar body extrusion and **b)** illustrates the meiotic spindle of the second polar body extrusion after spermatozoa insemination; where the mitotic spindle is seen after pronuclei undergo syngamy. Image sourced from: (Palermo *et al.*, 1997).

When the PNs start to rotate, the PN of the male and the female need to align. In the case of failed alignment of polar bodies and pronuclei, abnormal fertilization and cleavage can occur (Balaban *et al.*, 2011).

Aguilar *et al.* (2014) conducted a retrospective study to describe timings of fertilization events in implanted and non-implanted embryos and showed that timings of second polar body extrusion, pronuclear fading and the length of the S-phase was linked to implantation.

Azzarello *et al.* (2012) investigated whether PN morphology and PN fading could predict live birth outcome. Time of PN fading was calculated from time of insemination until PN breakdown. They showed that embryos with PN fading earlier than 20.45 hours post insemination did not result in live birth. They also reported that PN morphology changes over time and showed no significant difference in live birth.

Wirka *et al.* (2014) investigated the association with abnormal syngamy, abnormal first cytokinesis, abnormal cleavage, chaotic cleavage and embryo morphology and development, using the Eeva Time lapse system. They also investigated the syngamy timing defined as time from PN disappearance until time of first cell cleavage. They defined abnormal syngamy as the presence of disordered pronuclei movement and delayed dispersion of the nuclear envelopes. Results from the study showed that the mean syngamy time was significantly shorter for embryos displaying abnormal syngamy (1.8 ± 1.4 hours) in comparison to embryos without abnormal syngamy (2.4 ± 1.8 hours). It was also reported that embryos with abnormal syngamy had a significantly lower blastocyst formation rate (21.5%) in comparison to normal syngamy embryos (44.9%). In addition, only 30% of the abnormal syngamy embryos resulted in good or fair quality blastocysts in comparison to 60% of the normal syngamy embryos, which resulted in good or fair quality blastocysts.

Ueda *et al.* (2012) reported on the relationship between the timing of syngamy and human embryonic development using time-lapse cinematography. This study defined syngamy time duration as second polar body extrusion until pronuclear fading. They showed that syngamy time for IVF was 21.2 ± 3.6 hours and 20.8 ± 3.6 hours for ICSI, which had no significant difference. Good quality embryos had a syngamy time of 20.0 ± 3.3 hours, in comparison to poor quality embryos of 22.1 ± 4.3 hours, which was significant. Results also showed that abnormally fertilized embryos had longer syngamy times of 25.1 ± 6.3 hours, which showed a significant delay compared to normally fertilized embryos.

Early cleavage evaluation and syngamy determination varies greatly between laboratories (Balaban *et al.*, 2011). Many studies have shown that the first cell cleavage can predict embryo quality and implantation (Balaban *et al.*, 2011). Early and late syngamy timing or early cleavage could have a poorer prognosis.

Embryo cleavage observation schedule at optimal cleavage rates were determined at the Istanbul consensus workshop as: Day 1 (fertilization check 17 ± 1 hours post insemination and syngamy check at 23 ± 1 hours post insemination), 2-cells (26 ± 1 hours post-ICSI, 28 ± 1 hours post-IVF), Day 2, 4-cells (44 ± 1 hours), and Day 3, 8-cells (68 ± 1 hours) (Balaban *et al.*, 2011). Uneven cleavage is classified as abnormal, as it has been correlated to chromosome aberrations and multinucleation (Hardarson *et al.*, 2003).

The fact that the spermatozoa has such an important contribution to syngamy and possibly early embryogenesis, suggests that the spermatozoa play a role during fertilization, as well as embryo development. Some studies state that male factor infertility should further be investigated with regards to syngamy (Wirka *et al.*, 2014).

Recently, evidence has suggested increased epigenetic effects from the male spermatozoon, which may alter DNA methylation, non-coding RNA and histone modifications taking place during gametogenesis and early embryogenesis (Rodenhiser and Mann, 2006, Denomme *et al.*, 2018).

Sperm DNA inheritance and epigenetic imprinted DNA play a key role in fertilization and embryo development (Denomme *et al.*, 2018). Many studies link poor male diagnosis with abnormal epigenetic characteristics, although there is little evidence of what the effects thereof are after fertilization (Denomme *et al.*, 2018).

Castillo *et al.* (2018) conducted a review on the spermatozoa proteome in order to associate the spermatozoa's proteins with function during preimplantation embryogenesis. The review included the proteomes from the spermatozoa, oocyte and early embryo. Complicating the assessment of spermatozoa function is the fact that oocyte and early embryo biological matter and information is minimal, which restricts research on developing embryo proteomes.

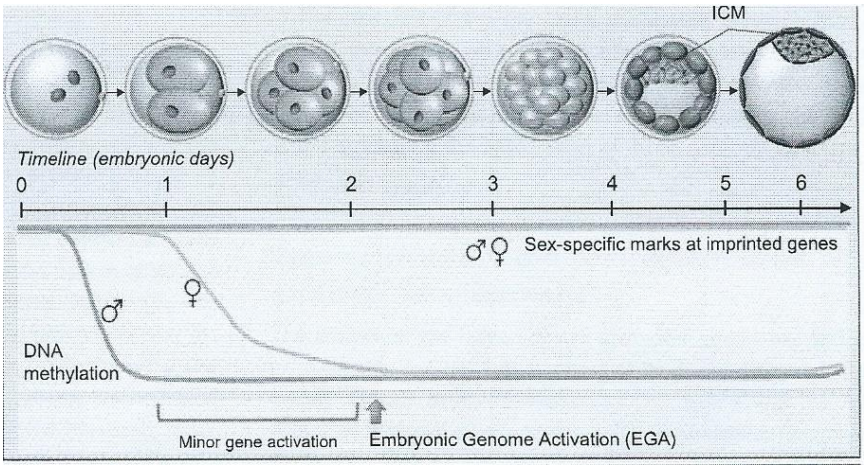

Figure 1.5. Time-line according to days of early embryo development after fertilization up to blastulation. Decline of DNA methylation of the paternal and maternal genes are indicated, as well as minor gene activation prior to the Embryonic Genome Activation. Sex-specific marks at imprinted genes are indicated at day 3 of development. Figure sourced from Castillo *et al.* (2018).

The fertilization process, as previously described, is an intricate process that involves the oocyte as well as the spermatozoa. The analysis of the sperm proteome identified 103 proteins which are involved in the fertilization process (Castillo *et al.*, 2018). After formation of the zygote, the chromatin undergoes many changes, such as replacement of male protamines with maternal histones. It has been determined that from the 2-cell stage up until the 4-8 cell embryo stage, one can observe some minimal gene activation. This transcription of genes might be due to the fact that DNA undergoes de-methylation, allowing gene activation and transcription into proteins. The embryo's own genome is only activated around the 4-8 cell stage, as depicted in Figure 1.5. The embryo synthesizes its own proteins from its genome, with many proteins possibly originating from the paternal or maternal RNA. Castillo *et al.* (2018) also observed some critical proteins which play a role during early embryogenesis that might be paternally derived. Knowledge about paternal originating proteins and their role during fertilization and pre-implantation embryogenesis is still very limited and needs to be studied further.

Denomme *et al.* (2018) studied the outcomes of patients with oligo-astheno-teratozoospermia (OAT), who had undergone preimplantation genetic testing (PGT) and euploid embryo transfer. They also looked at the global methylome and transcriptome of the blastocysts from these patients. They found that there was an increased miscarriage rate in OAT patients compared to normal male diagnosis patients. The methylation and transcriptome of OAT male factor patients resulted in aberrations which translate in epigenetic events that consequently lead to reduced reproductive potential of the embryo's associated with poor male sperm (Denomme *et al.*, 2018). It is clear that although this study supports other literature, indicating that the spermatozoon influences embryo competence, more in-depth studies are still required.

8. Blastulation and the role of male prognosis

In the ideal embryo development scenario, successful fertilization and syngamy is followed by the cleavage stages of embryo development, from 2 cells on day 1 to more than 8 cells on day 3, and continues to form a compacted morula and finally a blastocyst on day 5. The morula stage should be reached approximately 4 days after fertilization, which is when it enters the uterus (Moore *et al.*, 2011). A fluid-filled cavity starts to form inside the morula, known as the blastocoel. The blastocoel continues to expand as fluid is transported through the sodium transport channels into the cavity. This helps to maintain osmotic balance by incorporating water with the influx of sodium ions (Sepúlveda *et al.*, 2011). The expansion of the blastocoel, together with other mechanisms such as enzymatic digestion, leads to hatching or breaching of the zona pellucida (ZP), in order for the trophectoderm epithelium to come into contact with the endometrial lining for implantation of the embryo (Campbell and Fishel, 2015).

Incubation and medium advances, as used in TL systems, has facilitated extended embryo culturing periods up to blastocyst stage on days 5 and 6. Blake *et al.* (2007) performed a meta-analysis showing that day 5 blastocyst transfer in comparison to day 3 transfers improved pregnancy rates and live birth outcomes. The Glujovsky *et al.* (2012) Cochrane review reported an increased live birth rate for blastocyst culture, but no difference in clinical pregnancy or miscarriage rates between early cleavage and blastocyst transfers.

Many studies have been conducted on factors impacting blastocyst development. Westphal *et al.* (2003) investigated whether ICSI cycles had lower blastocyst formation and pregnancy outcome compared to conventional IVF. ICSI was used as insemination method for patients who had an indication based on abnormal semen parameters or previously failed IVF. Results showed no difference between ICSI and IVF with regards to blastulation (78% in IVF and 73% in ICSI) or viable pregnancy rates (51.4% in IVF and 55% in ICSI).

Sepúlveda *et al.* (2011) investigated the outcomes in IVF and ICSI cycles of different female age groups with regards to blastocyst formation, implantation, pregnancy and miscarriage. The groups were divided into oocyte donors (control group), <35 years, 35-39 years and >39 years. Results showed that all groups of patient ages had similar blastulation rates (40.2%, 37.2%, 34.2%) and only the control group had a different blastocyst formation (43.6%), pregnancy and implantation rate. They reported that patients over 39 years had a significantly higher miscarriage rate of 59.1% compared to the other groups (oocyte donors = 13.2%, <35 years = 12.7% and 35-39 years = 21.3%).

In addition to blastulation, the characteristics of the blastocyst is also of importance to determine quality and implantation potential in embryo selection. The process of blastulation results in a blastocyst consisting of the blastocoel, inner cell mass (ICM) and the trophectoderm epithelium (TE) layer, which can be distinguished from each other as seen in Figure 1.6.

The outer layers of the blastocyst form the TE, which is a thin layer of special epithelial cells giving rise to the embryonic placenta (Moore *et al.*, 2011). The centrally located blastomeres form the ICM, which gives rise to the foetus (Moore *et al.*, 2011).

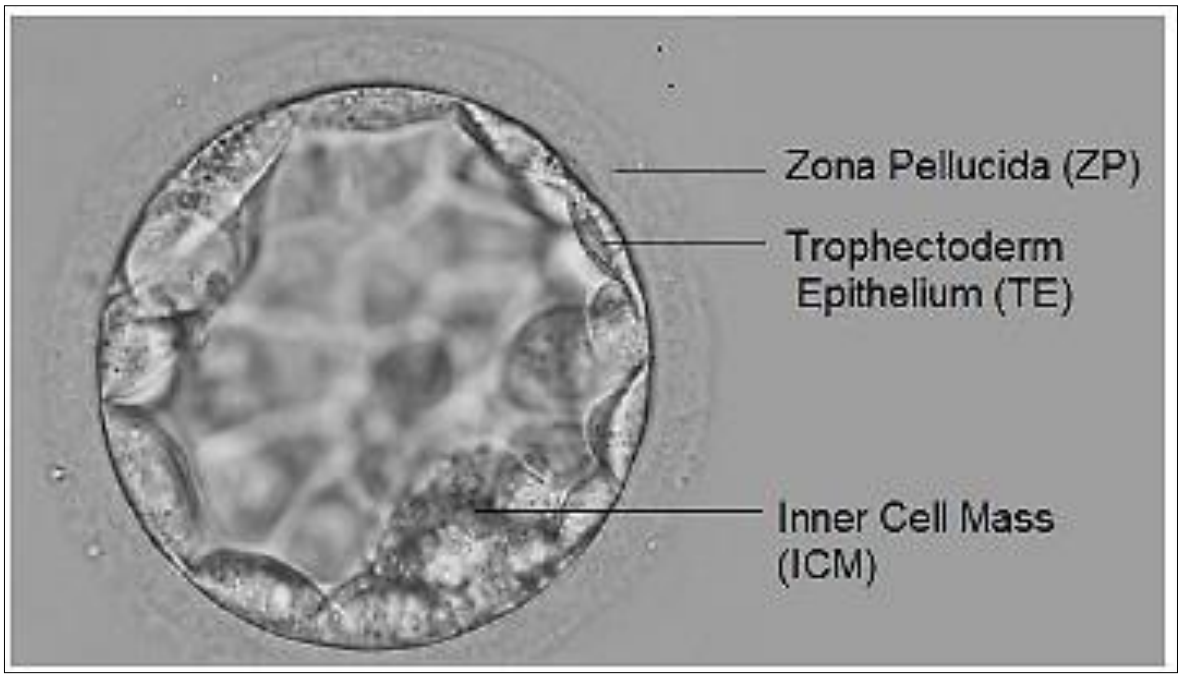

Figure 1.6. Example of blastocyst and features that are evaluated are indicated on the figure. Artwork by (Elder and Dale, 2010).

Grading of blastocyst morphology is the main method being used routinely for embryo selection and was first described by Schoolcraft *et al.* (1999). Many laboratories have based their embryo grading on the Veeck or Gardner scores (Schoolcraft *et al.*, 1999, Veeck and Zaninovic, 2003), of which this study uses a modified Gardner score (See Appendix G). The Gardner grading of the blastocyst expansion, TE and ICM is described below.

Blastocyst expansion:

1 – Blastocyst fills the perivitelline space (PVS), trophectoderm is aligned to the zona pellucida (ZP), where the ZP is still its original thickness.

2 – Blastocyst begins to expand and ZP is two thirds its original thickness.

3 – ZP is one half to one third its original thickness.

4 – ZP is very thin, but no hatching is visible yet.

5 – ZP is torn, trophectoderm bulges out of ZP.

6 – Blastocyst is more than 50% hatched out of the ZP.

Inner Cell Mass (ICM):

A – Many cells, tightly packed together, with no granularity.

B – Some cells, packed looser, with slight granularity.

C – Few cells, very loosely packed, granular and dark.

Trophectoderm epithelium (TE):

A – Many cells (>80%) epithelial cells with clear nuclei, which multiply during expansion, no granularity.

B – 50 – 75% Epithelial cells, some areas have stretched TE, few granular areas.

C – TE are few and stretched, with granular and dark areas.

Many studies have been conducted to evaluate the effect of the three blastocyst parameters on ART outcomes such as implantation and pregnancy.

Desai *et al.* (2016) reported on which blastocyst features independently predicted successful pregnancy and live-births from vitrified-warmed embryos. Results showed that blastocyst expansion grade was predictive of successful outcomes irrespective of ICM or TE. However, in their univariate analysis, ICM and TE grading also correlated positively with abovementioned outcomes.

Du *et al.* (2016) conducted a similar study to Desai *et al.* (2016), investigating the effect of all three parameters of blastocyst grading. Their findings showed the degree of blastocoel expansion was predictive of live birth in fresh and vitrified-warmed single embryo transfer. ICM grade was significantly related to live birth in fresh cycles when using a univariate mode, but was not seen in the multivariate logistic analysis. Neither ICM nor TE was correlated with live birth using logistic regression analysis. Their conclusion was that blastocoel expansion was a better predictor of live birth than ICM or TE.

Zaninovic *et al.* (2001) conducted a retrospective study to correlate blastocyst expansion, ICM or TE quality as separate and individual parameters to implantation. They found that there was no significant difference between degree of blastocyst expansion or ICM morphology and implantation rate, however, they showed a significantly higher implantation rate when an embryo with an A-grade trophectoderm (76%) was selected for transfer. The B- and C-grade trophectoderm embryos transferred had a lower implantation of 56% and 50% respectively. In addition, the study reported that an A-grade TE morphology correlated with the survival of blastocysts after thawing, compared to B- and C-grades (85%, 63% and 62% respectively). It was concluded that TE has a greater impact on implantation than blastocyst expansion and ICM quality, attributed to the fact that the TE plays a critical role during attachment to the uterine wall.

Ahlström *et al.* (2011) and Hill *et al.* (2013) both did retrospective studies to investigate each blastocyst morphological parameter and the relation to live birth outcome. The results of both studies showed that TE had a stronger correlation to implantation and live birth outcome than ICM. Similar results were shown more recently by Ebner *et al.* (2016) who conducted a prospective study to determine whether ICM size and quality, TE morphology and cell number, or expansion correlated with ART outcome in both fresh and frozen embryo transfer cycles. Results showed that TE quality and cell number was the strongest predictor of treatment outcome, and correlated with live birth and miscarriage. Therefore, they suggested that TE be prioritised before ICM grading during embryo selection.

Thompson *et al.* (2013) also did a retrospective study on SET cycles to determine which blastocyst morphology parameters better predicted CPR and LBR. They categorized expansion into early, expanded and hatching blastocysts. They categorized ICM and TE into poor, fair and good scorings. Based on their results, it was concluded that better trophectoderm morphology and further blastocyst progression showed higher CPR and LBR, therefore being the most important parameters to use when selecting an embryo for transfer. ICM did not result as a predictive indictor of CPR or LBR. Younger patients also showed higher CPR and LBR.

TL technology allows embryologists to observe cleavages and the start of blastulation (tSB), which is annotated as the time point where the first sign of formation of a blastocoel cavity is observed (Campbell and Fishel, 2015). Some of the early embryo kinetic parameters have been studied using TL to predict blastulation.

A study done by Wong *et al.* (2010) identified a combination of three cleaving morphokinetic data parameters i.e. first cell cytokinesis duration, time between the first mitosis completion and second mitosis initiation and the time duration between the second and third mitoses, to predict blastocyst formation using their algorithms before day 4 of embryo development and reported a 93 to 94% sensitivity and specificity.

Lemmen *et al.* (2008) investigated the events after fertilization to identify markers that could be correlated to good quality embryos on day 2 and implantation. Their results showed correlations between earlier PN disappearance after fertilization, earlier first cell cytokinesis and increased number of blastomeres on day 2 of embryo development. They also showed correlations with pregnancy rate when nuclei appeared synchronously after first cell cytokinesis, of which time range references were not reported. Two previously discussed studies showed contradictory findings compared to that of Lemmen *et al.* (2008). Azzarello *et al.* (2012) showed no live birth when zygotes had too early PN fading post insemination (20.45 hours). Meseguer *et al.* (2011) also showed that first cell cytokinesis taking place too early could be detrimental to implantation.

Desai *et al.* (2014) investigated the time differences of kinetic cell divisions between embryos forming blastocysts and their potential for implantation using the EmbryoScope™. Kinetic time points from insemination was assessed using TL, which included time to syngamy (tPNf), t2, time to two cells (c), 3 cells (t3), 4 cells (t4), 5 cells (t5), 8 cells (t8), morula (tM), start of blastulation (tSB); time to blastocyst (tBL); expanded blastocyst (tEBL). They also looked at the duration of the second (cc2) and third (cc3) cell cycles, the t5-t2 interval and the time duration of synchronous divisions s1 (t2 - tPNf), s2 (t4 - t3) and s3 (t8 - t5). Results showed that tPNf, t2, t4, t8, s1, s2, s3 and cc2 were significantly different in embryos forming blastocysts compared to embryos either failing to reach blastulation or forming poor quality blastocysts.

Time to syngamy, defined as time from ICSI to PN fading, was also investigated in relation to blastulation. Time to syngamy was 24.8 ± 2.6 hours in good quality blastocysts selected for transfer of which implanted blastocysts showed a significantly shorted syngamy timing of 24.1 ± 2.5 hours compared to 26.2 ± 2.7 hours in those that did not implant. Time to syngamy was significantly longer with 26.8 ± 8.3 hours in poor quality and non-blastulating embryos compared to the transferred blasts and frozen blastocysts (25.2 ± 3.0 hours).

Their comparisons of embryo morphokinetics in implanted embryos and non-implanted embryos suggested that embryo kinetic markers may differ in the ability to reach blastocyst stage. They also reported a CPR of 72% and an implantation rate of 50% in their study.

Milewski *et al.* (2015) also used morphokinetic parameters from TL EmbryoScope™ incubator culture to predict blastocyst development. They developed a predictive multivariate model algorithm from timings of two- and five cells and intervals between second and third division. They concluded that such a model can enable embryologists to identify embryos with high reproductive potential earlier in embryo development and can subsequently shorten embryo incubation.

Knowledge of the role and functions of the **spermatozoa** during early embryo development and blastulation is still controversial, as data is challenging to assess.

Desai *et al.* (2009) investigated the paternal effect from epididymal and testicular sperm sources by looking at zygote formation, embryonic cleavage and genomic activation. The paternal genome activation in ICSI cycles were evaluated by using embryonic compaction and blastulation as indication of zygotic transcription onset, which occurs at the 4 to 8-cell stage, as mentioned before. Results showed no difference in compaction or blastulation rates, respectively, amongst epididymal (74%, 38%), obstructive azoospermic testicular (69%, 42%) or non-obstructive azoospermic testicular (79%, 38%) spermatozoa. The only parameter significantly affected in non-obstructive azoospermic testicular cases was the fertilization rate.

French *et al.* (2010) did a retrospective review to investigate whether Tygerberg strict criteria correlates with outcomes in ICSI cycles. They divided the sperm morphologies into subgroups: 0%, 1%, 2%, 3%, 4%, 5–7%, and >7% normal sperm morphology. They did not observe any difference in blastulation rate amongst the groups, which was from 41% to 50%. They found that fertilization, implantation, pregnancy and live birth rates were not statistically different between the groups and no effect was observed on blastocyst development or quality. Surprisingly, the study results showed a higher percentage of good quality blastocysts in the most severely teratozoospermic group with 0% normal forms in comparison to patients with ≥5% normal forms (37% vs. 28%). They attributed this outcome to patient etiology distribution with less prevalence of female factor infertility in the severely teratozoospermic group.

Hickman *et al.* (2013) investigated the effect of sperm origin, maternal age and embryo ploidy on embryo morphokinetics using TL. Comparisons were made between surgically retrieved and ejaculated sperm, embryos from older (≥37 years) and younger (≤36 years) female patients, and genetically abnormal and normal chromosome screening of embryos. They showed that sperm source and maternal age contributed to embryo morphokinetics. Embryos resulting from surgically retrieved spermatozoa had delayed morulation, and blastocyst hatching, but had a shorter duration until two cell stage, compared to those from ejaculated sperm. Younger patients reached morulation, expanded and hatching blastocyst stages faster than older patients. Lastly, morphokinetics were not affected by ploidy status. They concluded that sperm source and maternal age contributes to embryo morphokinetics, suggesting the role of the spermatozoa in the first cleavage and embryo development after embryonic genome activation.

Bartolacci *et al.* (2018) conducted a retrospective analysis to investigate the impact of male age, spermatozoa concentration and motility on blastulation rate, fertilization rate, top quality blastocyst formation rate and pregnancy rate in ICSI cycles. They divided the male groups into four according to concentration based on the WHO criteria (2010). Results showed a significant difference in fertilization and blastulation rates, but no difference in top quality blastocyst formation rate or ongoing pregnancy rates. Blastulation rates were defined as the number of blastocysts formed per oocyte cultured and differed from the poorest group (spermatozoa concentration < 1 $x10^6$ per ml): 50.0 (33.3–66.3%) compared to the better group (spermatozoa concentration ≥ 15 $x10^6$ per ml): 55.6 (40.0–75.0%).

Mazzilli *et al.* (2017) investigated the effect of male factor on the outcomes in ICSI cycles in combination with pre-implantation genetic testing for aneuploidies (PGT-A). They divided the cycles into five groups according to male spermatozoa parameters: normozoospermia, moderate male factor, severe oligo-astheno-teratozoospermia, obstructive azoospermia, and non-obstructive azoospermia. Results showed a significant difference in fertilization rate between normozoospermia (77.2%) and all four other groups (74.8%, 68.7%, 67.3% and 53.1% respectively). Blastulation rate per fertilized oocyte differed significantly in the non-obstructive azoospermia group (40.6%) compared to the normozoospermia group (49.3%). The moderate male factor (48.6%), severe oligo-astheno-teratozoospermia (48.0%), and oligo-asthenozoospermia (45.5%) groups showed similar blastulation rate to the normozoospermia group (49.3%). The timing of blastocyst development was affected by obstructive and non-obstructive azoospermia. There was no correlation found between male factor and euploidy rate or perinatal outcomes.

Burruel *et al.* (2014) investigated how the patterns of blastocyst formation was affected by oxidative damage to spermatozoa by evaluating the duration between the first mitosis and start of the second mitosis (P2) in embryo development. They used the rhesus macaque as an animal model. The results showed that less embryos with short P2 times (<1 hour) reached blastocysts, and nearly all of the embryos with longer P2 times reached blastocysts by day 6 ($P < 0.01$). They also observed that duration of the second to third mitoses were sensitive cleavage phases when oxidative stressed spermatozoa were used. Embryos that had either too long or too short cytokinesis resulted in unsuccessful blastulation. Therefore, they suggested that paternal factors play a role in early mitosis, although paternal-derived genes are expressed during advanced embryo development.

Lammers *et al.* (2015) investigated the effect of sperm origin, i.e. either freshly ejaculated or surgically (testicular and epididymal) retrieved spermatozoa, on embryo morphokinetic parameters during ICSI cycles. They also compared clinical outcomes and showed that these were comparable between the two different sperm sources. Early morphokinetic parameters were comparable between groups, but the t3, t8 and s2 parameters showed significant differences. They concluded that there was no difference in morphokinetic analysis between fresh ejaculated and surgically retrieved sperm, but stated that more studies are required with regards to relationship between sperm origin and late morphokinetic parameters such as blastocyst development.

Desai *et al.* (2018) investigated the influence of spermatozoa sources on kinetic embryo development and clinical outcomes. The groups were divided into epididymal, testicular (obstructive and non-obstructive azoospermia diagnoses) and ejaculated normal spermatozoa. Results showed no difference in CPR between the three groups and a non-significant decline in implantation and birth rate of testicular spermatozoa compared to epididymal spermatozoa and the control group. They did see lower compacting embryo rates in non-obstructive testicular spermatozoa compared to the others, which is indicative of paternal activation (Desai *et al.*, 2009). In the testicular sourced spermatozoa group, a significantly lower percentage of embryos showed kinetics typically attributed to high quality embryos. Their conclusion was that TL allows for greater comparison of paternal influence on embryo morphokinetics.

9. Conclusion

Specific morphokinetic events during embryo development after ART procedures have been used as markers for embryo selection, such as syngamy timing, to determine the viability and potential of successful embryo development and implantation (Mastenbroek *et al.*, 2011, Wirka *et al.*, 2014). Time lapse technology has added information leading to the development of algorithms (Fishel *et al.*, 2018) to further improve the selection of embryos. However, patient diagnosis has not yet been taken into consideration in these algorithms. Neither the effect of male prognosis or the effect of syngamy timing on day 5 blastocyst development and quality have been elucidated.

The role of spermatozoa and oocytes in syngamy, one of the key events during fertilization, and subsequent blastulation, which is a major predictive indicator of implantation, are still to be elucidated. This study aims to determine whether syngamy timing differs according to male patient prognosis and whether this in turn also affects the advanced embryo at blastocyst stage.

RESEARCH QUESTION

Is there a relationship between syngamy timing, blastulation rate and blastocyst quality according to different male fertility prognosis groups in ART treated patients?

HYPOTHESIS

H0 (Null hypothesis) = There is no association between fertilization rate, syngamy timing, blastulation rate or blastocyst quality and male factor fertility prognosis.

H1 (Alternative hypothesis) = There is an association between fertilization rate, syngamy timing, blastulation rate or blastocyst quality and male factor fertility prognosis.

AIMS AND OBJECTIVES

1. Primary aim

The primary aim of this study was to investigate the possible measurable direct effect of male fertility diagnosis on the time duration to the syngamy event when the spermatozoon contributes essential centrosomes during fertilization.

2. Secondary aim

The secondary aim was to investigate the sequential effect of male fertility diagnosis on syngamy timing and resulting advanced embryo development when the paternal genome actively contributes to blastocyst formation and quality.

3. Objectives

- To identify and categorize treatment records into male prognosis groups according to diagnosis criteria:
    - Good prognosis, i.e. normal sperm diagnoses;
    - Sub-fertile prognosis, borderline diagnoses spermatozoa and
    - Severe sub-fertile prognosis, severe male factor diagnoses.
- To calculate syngamy timings by using TL morphokinetic data points (tPNf - tPB2).
- To determine the fertilization and blastulation rates of all the groups by using TL.
- To determine and compare syngamy timing between the 3 male prognosis groups using TL.
- To correlate overall syngamy timing with blastocyst quality parameters on day 5 and also between the 3 male prognosis groups using TL.
- To determine whether syngamy timing could contribute to day 5 blastocyst prediction and better embryo selection methods.

CHAPTER 2

MATERIALS AND METHODS

1. Study design

This was a retrospective study on embryo development records collected during standard embryo time-lapse evaluation after IVF and ICSI insemination treatment. No other interventions were performed. The study was completed over a period of two years, 2017 to 2018.

Non-identifiable morphokinetic data of embryo development was extracted and sorted according to three defined male prognosis groups. The main parameter investigated and compared between the three male prognosis groups was syngamy timing. The secondary investigation focussed on both syngamy timing and subsequent blastocyst quality 116 to 125 hours post insemination, between the three male prognosis groups, as well as overall.

2. Study population

All oocytes arising from patients who received any of the following ART procedures: IVF, ICSI, PICSI, and IMSI treatment from 2013 to 2016, at Wijnland Fertility Clinic in Stellenbosch, that were incubated in the time-lapse incubator, were included in the initial data set. All female diagnoses and age groups were included. Patient information was kept strictly confidential.

The exclusion criteria are listed in the section (3) below. These criteria were specifically used in order to remove apparent major bias factors that could have had an impact on the outcomes of the study.

3. Exclusion criteria

The following exclusion criteria was applied to exclude irrelevant records to reduce statistical noise and possible confounding clinical patient factors. There also were records excluded due to unforeseen circumstances such as embryo developmental arrest.

3.1 To reduce statistical noise

Fertilization and initial data set phase:

- Immature oocytes: Metaphase I (MI), germinal vesicle (GV) or atretic oocytes before insemination.

Syngamy Timing calculation phase:

- Zygotes with no or abnormal fertilization – 0PN, 1PN or ≥ 3PN or degenerative.

Blastulation calculation and grading phases:

- Zygotes with no or abnormal fertilization – 0PN, 1PN or ≥ 3PN or degenerative.
- Zygotes not reaching either compact morula or blastocyst stage on day 5 of embryo development.

3.2 To reduce possible additional confounding factors

- Cancer patients with previous or current diagnosis due to possible unknown effects of cancer or cancer treatment on gametes.
- Human Immunodeficiency Virus (HIV) or Sexually Transmitted Infections (STI) / sero positive patients - due to very low numbers and the possible effects of infections and/or anti-retroviral medication on fertility, gametes and embryo development that have not been elucidated.

3.3 Inevitable exclusions due to unforeseen / unavoidable circumstances

There is a natural drop-out trend of oocytes, zygotes and embryos in ART cycles due to developmental arrest. This was clearly observable in the sample size reduction from oocytes, to zygotes, to blastocyst development stage in this study.

Records with any missing data points resulting from any of several reasons, for example human error or visual difficulty during annotation, were inevitably excluded.

4. Sample size

The initial population size of N = 3103 mature oocytes were available in the raw data set. A total of 1292 oocytes had abnormal or failed fertilization and were excluded after fertilization rate calculation. The total number of normally fertilized (2PN) oocytes, that resulted in zygotes after fertilization, were 1811.

After exclusion of 223 zygotes with missing tPNf and tPB2 data points, the final number of zygotes available for syngamy timing calculations, were n = 1588.

A total number of n = 1262 embryos successfully reached compact morula stage from the 1811 normally fertilized zygotes. There were 1209 embryos that initiated blastocyst formation, with a tSB data point available to calculate blastulation rate.

The number of embryos with day 5 development stage or expansion grade available for investigation were n = 1262 which included compact morulae (n = 53) and blastocysts (n = 1209). The number of expanded blastocysts with available trophectoderm and ICM scores were n = 857.

5. Division into male prognosis groups

All males were included and categorized into three specific prognosis groups according to semen analysis diagnoses at the time of ART. The three male prognosis groups are specified as: **good prognosis group (GP)**, **poor prognosis group (PP)** and **very poor prognosis group (VP).** Refer to Appendix A for Diagnoses definitions.

Good Prognosis group (GP) diagnosis criteria:

Cases where either a) or b) were applicable:

a) Normal concentration of ≥ 15 x10^6 per ml, and normal motility of ≥30% with either:
    - N-pattern morphology diagnosis - which is defined as a morphology result of greater or equal to 15% **or**,
    - G-pattern morphology diagnosis - which is defined as a morphology result of 5-14%.
b) Donor sperm:
    - Sperm concentration ≥ 80 x 10^6 per ml, motility ≥ 50%, morphology ≥ 8%, **and/or**,
    - Consideration of appealing demographic profile.

Poor Prognosis group (PP) diagnosis criteria:

Patients with a poor prognosis were either diagnosed with a) or b) or c):

a) Normal sperm concentration of ≥ 15 x10^6 per ml, and normal motility of ≥30% with:
    - P-pattern morphology diagnosis - which is defined as a morphology result of 1-4%, also known as teratozoospermia **or**,
    - Borderline morphology diagnosis (GP-pattern) - which is a morphology pattern that falls into both the good and poor pattern categories (for example 4% and 5% morphology determined by two embryologists, respectively).
b) Oligozoospermia (5 - 14 x10^6 per ml), irrespective of morphology diagnosis.
c) Vasovasostomy aetiologies.

Very Poor Prognosis group (VP) diagnosis criteria:

Patients with a very poor prognosis were either diagnosed with a), b), c) or d):

a) Astenozoospermia – characterized by semen samples with:
    - motility of less than 30% **and / or**,
    - forward progression of less than 2.

b) Severe oligozoospermia (<5 x10^6 per ml), irrespective of morphology diagnosis.
c) Double or triple sperm defect diagnoses, where a combination of more than one abnormal sperm parameter is present, for example: oligo-asteno-teratozoospermia (OAT).
d) Azoospermia, including obstructive, non-obstructive, and vasectomy, where spermatozoa were retrieved via testis biopsy procedure.

6. Data management

Data for the project was acquired by exporting the data file that was automatically generated by the EmbryoScope™ system during embryo annotation. The data of selected years (2013 to 2016) were exported into an excel spreadsheet (*see Appendix C*).

The retrospective data was verified to reduce discrepancies and missing data points as far as possible. The records were then de-identified and aggregated to only collect the relevant medical diagnosis data and laboratory embryo annotations as the final dataset for analysis. It was subsequently filtered according to inclusion and exclusion criteria and finally divided into male prognosis groups as described. The applicable data columns required to perform the analyses and calculations were utilized, which included female age, female primary diagnosis, male diagnosis, tPNf, tPB2, PN status, start of blastocyst formation (tSB), and blastocyst quality (BQ) grade.

The fertilization rate, syngamy timing, and blastulation rate were mathematically calculated.

Patient diagnoses data was used for descriptive analysis of the sample size. The average female patient age was calculated per treatment cycle for each male prognosis group.

The time duration to syngamy of the normally fertilized oocytes post insemination was calculated by subtracting the time of PN fading from the time of second polar body extrusion: Syngamy timing = tPNf – tPB2. The blastocyst formation rate was defined as the total number of blastocysts among the total normally fertilized (2PN) oocytes (Sepúlveda *et al.*, 2011). Blastulation rate was calculated as a percentage value by dividing the number of early cavitating blastocysts (reached tSB) on day 5 of embryo culture by the total number of 2PN zygotes (normally fertilized oocytes): Blastulation rate = total embryos with tSB / total number of 2PN zygotes.

Blastocyst quality (BQ) on day 5 was evaluated as described in the methodology section. Male fertility prognosis groups were correlated to syngamy timing, blastulation rate and BQ.

7. Assisted reproductive technology methods

All patient diagnosis assessments and ART treatments were done according to the standard established practices of Wijnland Fertility Clinic. The assessment of female diagnosis was done by specialists at Wijnland Fertility Clinic according to established diagnostic criteria (*see Appendix A*). The ART treatment procedures which included controlled ovarian stimulation, oocyte retrieval, semen processing and *in vitro* fertilization were done according to standard operating procedures (SOP) of Wijnland Fertility Clinic (*see Appendix G*).

The most relevant procedures to this study are discussed in more detail below.

8. Semen analysis and diagnosis

The basic semen analysis entails parameters such as spermatozoa concentration, motility and morphology. This diagnostic test is done in advance to ART treatment and plays an important role in deciding on the specific ART treatment to follow. Therefore, the test may be repeated to achieve accurate results before final diagnosis and prognosis is established.

Concentration and motility are determined by investigating a wet preparation slide of the semen sample. At least 10 fields are assessed to estimate concentration ($x10^6$ per ml) and motility (percentage). This is made by placing a drop of semen onto a glass slide with a glass cover slide over. The morphology parameter is determined by making a smear of the semen sample on a glass slide and staining it with Hemacolor® (Merck™, South Africa), also known as the Diff-Quik method. The unmounted morphology slide was then examined under a light microscope under oil at 1000x times magnification and 100 spermatozoa are evaluated as either normal or abnormal based on the Tygerberg strict criteria (WHO, 2010). Morphology was also expressed as percentage.

The normal values for the following parameters were: concentration ≥ 15 x 10^6 per ml, motility ≥ 30%, forward progression >2 and morphology >4%. Diagnosis is based on these parameters, as well as other macroscopic parameters such as volume and pH. The male diagnosis for each patient was based on the sample parameters of the sample used on the day of the ART procedure. Definitions of diagnoses can be found in Appendix A and Wijnland Fertility Andrology reference values (*see Appendix B*).

9. Time-lapse incubation equipment: EmbryoScope™

9.1 Time-lapse EmbryoScope™ incubator

The EmbryoScope™ incubator is designed to provide optimal culture condition as well as embryo monitoring and annotation (*See Fig 2.1*). All electronic components are kept in a separate compartment ensuring no embryo exposure to electric fields during culturing and incubation.

The incubator allows embryo culturing in small individual wells and has improved carbon dioxide (CO_2) and oxygen (O_2) recovery rates compared to bench-top incubators.

The incubators have a tri-gas system and gases are re-circulated through a HEPA and carbon filter to minimize volatile organic compounds and other particles. Temperature of the EmbryoScope™ incubator is kept constant and the heat foil inlay heating system is mounted within the slide holder core. Vitrolife performed temperature experiments and concluded that each micro-well differed approximately 0.2°C from the heated core. Therefore, temperature of the heated core should be about 37.2°C in order to achieve 37.0°C.

Time-lapse EmbryoScope™ incubators are not humidified and require oil-overlaid culturing. Non-humidified incubation has some benefits which include minimizing contamination of fungal growth, airborne particles and pathogens. Oil-overlay promotes minimizing microenvironment fluctuations, by delaying gas diffusion and evaporation. Vitrolife also performed experiments on osmolality after 5 days of culturing and established that the osmolality of the media is within the range for human embryo culture. If any changes in pH occur, oil will increase equilibration time. Imaging in the EmbryoScope™ is acquired by using a single red LED (635nm). Light exposure is controlled by built-in monitors, ensuring that defined limits are not exceeded.

Figure 2.1. The EmbryoScope™ Time-Lapse system with incubator and Software.
Image sources from Vitrolife Technotes: https://www.vitrolife.com/globalassets/support-documents/tech-notes/technote_safety-of-incubation-in-the-embryoscope-time-lapse-system.pdf

9.2 Time-lapse embryo culture dish

EmbryoSlide® culture dishes are specifically designed for the EmbryoScope™ time-lapse incubators to ensure safe handling, accommodate single embryo culture and allow accurate and high-quality microscopy imaging of each embryo. Each EmbryoSlide® is produced from culture-tested polystyrene and has 12 microwells that allow for culturing of up to 12 embryos, as well as 4 wash wells. EmbryoSlide® culture dishes were prepared either on the day before embryo culture and equilibrated overnight or on the day of use and pre-equilibrated ≥4 hours before embryos are incubated for culturing. EmbryoSlides® were prepared with cold universal embryo culture medium and oil, on a non-heated workbench. Each microwell was filled with a small amount of medium to create a meniscus, thereafter filled completely with 25ul of medium and approximately 1.4ml of oil is then overlaid (*see Appendix G*). Bubbles which may have formed after equilibration of ≥4 hours are removed with a micropipette (*see Appendix G*).

After insemination, oocytes were placed in the EmbryoSlide® to be cultured in the EmbryoScope™ incubator for 6 days and annotations were made during this period.

10. Embryo annotation and syngamy

Each patient's embryos were labelled on the system with patient information which included female age, diagnosis, and case number for accurate matching of patients to their embryos. Each embryo culture slide was then also automatically allocated a unique embryo slide identifier and an embryo identification number which was stored in the background by the system. These identifiers were used to distinguish between treatment cases and individual embryos. The embryos were not for embryo evaluation as all annotations and grading was done through the EmbryoScope™ image capturing software. The EmbryoScope™ ensures non-invasive evaluation and selection of the embryos.

The time-lapse incubator (EmbryoScope™) records images of each embryo at 15-minute intervals. Zygote and embryo development were annotated daily by embryologists at certain embryo development stages. Specific fertilization annotations include time of second polar body extrusion (tPB2), the time of PN appearance (tPNa), the confirmed number of PN's visible at 17 to 18hours (fertilization status), and time of PN disappearance (tPNf). Subsequent timings of embryo cell cleavages were also annotated (t2-t8) until 9-cell stage (on day 3 of embryo development). Advanced embryo developmental stages, such as morulation (tM) and blastulation was annotated from day 4 onwards, which included start of blastulation (tSB), full blastulation (tB), blastocyst expansion (tEB), hatching (tHB) and the final blastocyst grade. Vitrolife provides a detailed guideline of Time-lapse annotations and timings (*see Appendix G*).

11. Embryo evaluation – specifically blastocyst stage grading

Blastocyst evaluation for morphology grading was followed using the SOP of Wijnland Fertility Clinic (*see Appendix G*). Blastocyst scoring was done using a modified Gardner score in combination with the Vitrolife blastocyst scoring guidelines for the EmbryoScope™ (*see Appendix G*). These evaluations are based on the expansion of the blastocyst (scored 1-6), the inner cell mass (scored A-C) and trophectoderm epithelium (scored A-C). An A-grade ICM and TE is the best score and C-grade is subsequently the lowest.

The timing annotations and final blastocyst scores allow embryologists to select embryos for transfer, cryopreservation or rejection accordingly. The system also enables the exportation of the captured electronic data which contains every embryo's data points from the annotations and the embryo grading scores.

12. Flow diagram of data management

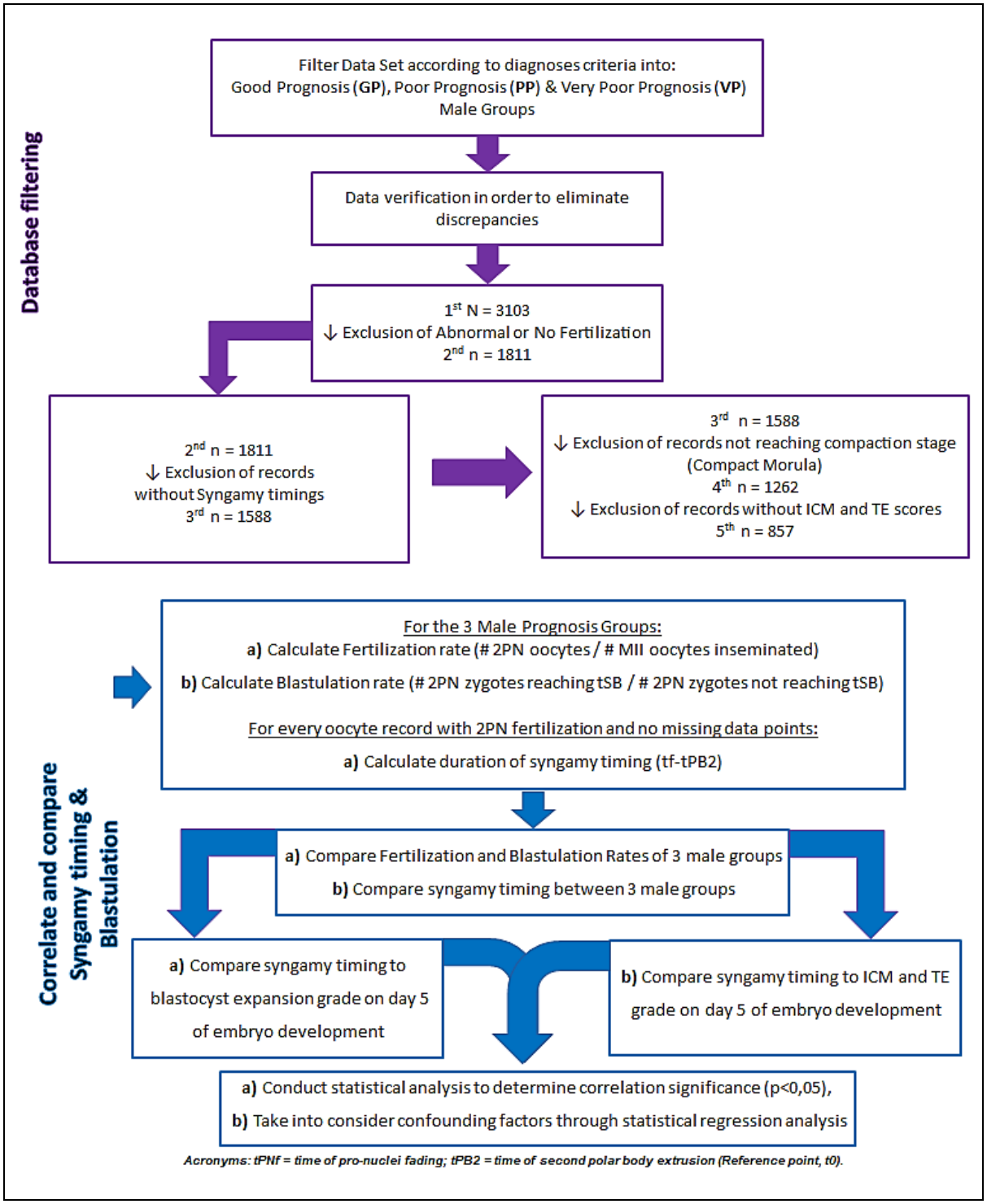

Figure 2.2. Flow diagram of data management and processing. **Phase 1** = Database filtering; **Phase 2** = Correlate and compare syngamy time and blastulation.

13. Limitations

One maternal factor limitation of this study is the heterogeneity of the female population. This was due to the fact that no female factor was excluded in order to ensure a powered sample size. Therefore, the maternal effect, apart from female age, which was compensated for, could not be excluded.

Sperm morphology is a subjective evaluation parameter; however, the reliability of this result is usually internally validated by peer-assessment. Internally, usually more than 100 spermatozoa are counted by more than one technician, although there is a possible discrepancy that can occur within laboratories. Therefore, external quality control is implemented to uphold benchmark standards. However, in this study morphology was only internally controlled and therefore borderline results remained in the dataset, with the lower morphology used as indicator for prognosis group categorization.

Blastocyst grading is also a subjective interpretation by embryologists (scientists and technologists) and therefore may affect consistency (reliability) of blastocyst gradings. However, the data set in this study contained data points that were evaluated by more than one embryologist at the time of annotation or grading (double witness system). IVF and ICSI insemination methods were both included. IVF sperm selection is based on natural sperm-oocyte binding interaction. ICSI is designed to compensate for poor male prognosis sperm and sperm selection is based on embryologist subjectivity

14. Ethical considerations

Ethical approval was obtained prior to study initiation for the period of: 09 February 2018 until 08 February 2019 for Project ID: 0751, HREC Reference: S17/08/158 (*see Appendix F*).

Due to the retrospective nature of the study, patients were not subjected to any additional risks, injury or pain. Data was anonymized and aggregated during importation into the work sheet for analysis, ensuring no patient identity was revealed, and remained confidential. The data was analysed in groups, ensuring that data was not analysed individually. The research was done to produce valuable information that likely could improve embryo selection and patient treatment in future.

The Health Research Ethics Committee kindly waived informed consent when the protocol was submitted for ethical approval.

15. Statistical analysis

A statistician from Stellenbosch University was consulted for appropriate statistical analysis and results.

For comparisons of the different diagnoses between groups, cross tabulation with the Chi-square test were done. Continuous variables like syngamy timing were compared between groups using mixed model ANOVA. In these analyses group and female age (covariate) were treated as fixed effects, and patients as random effect. Binary outcomes (e.g. fertilization) were compared between groups using generalized estimating equations (GEE) with the Binomial as underlying distribution. Age was included as covariate. For post hoc testing, Fisher Least Significant Difference (LSD) was used. The significance level of 5% (P <0.05) was used as guideline for determining statistically significant results.

CHAPTER 3

RESULTS

The results of the study are presented in Figures 3.1-3.13 and Tables 3.1-3.10.

1. Descriptive results of sample size

The PP group constituted the majority of the total sample size (n = 1588), consisting of 46.9% with 746 zygote records. The GP group consisted of 537 zygotes, which was 33.8% of the total sample size. The VP group had 305 zygotes and consisted of 19.2% of the total sample size (*see Table 3.1*).

The majority of the GP group zygotes had a paternal diagnosis of normozoospermia (90.7%) and the PP group mostly teratozoospermia (87.5%). The VP group mainly had non-obstructive testis biopsy diagnoses (21.3%) and oligoastenosoospermia (18.0%). The remainder of diagnoses in each group is further described in Table 3.1 and Figure 3.1.

Table 3.1. Descriptive table depicting the frequencies and percentage distribution of male diagnoses, overall and in each male prognosis group, which had normal (2PN) fertilization and available syngamy timings.

GOOD PROGNOSIS (GP) GROUP	**N**	**PERCENTAGE (%)**
Donor sperm	50	9.3
Normozoospermia	487	90.7
Total	**537**	**33.8**
POOR PROGNOSIS (PP) GROUP	**N**	**PERCENTAGE (%)**
Teratozoospermia	653	87.5
Oligozoospermia	40	5.4
Borderline Morphology	15	2.0
Oligo-teratozoospermia	36	4.8
Vasovasostomy	2	0.3
Total	**746**	**46.9**
VERY POOR PROGNOSIS (VP) GROUP	**N**	**PERCENTAGE (%)**
Severe Oligozoospermia	26	8.5
Asthenozoospermia	34	11.1
Astheno-teratozoospermia	21	6.9
Oligo-asthenozoospermia	55	18.0
Severe Oligo-asthenozoospermia	26	8.5
Severe Oligo-teratozoospermia	11	3.6
Oligo-astheno-teratozoospermia	45	14.8
Testis Biopsy – Non-obstructive	65	21.3
Testis Biopsy -Obstructive	11	3.6
Testis Biopsy – Post Vasectomy	11	3.6
Total	**305**	**19.2**
Grand Total	**1588**	**100**

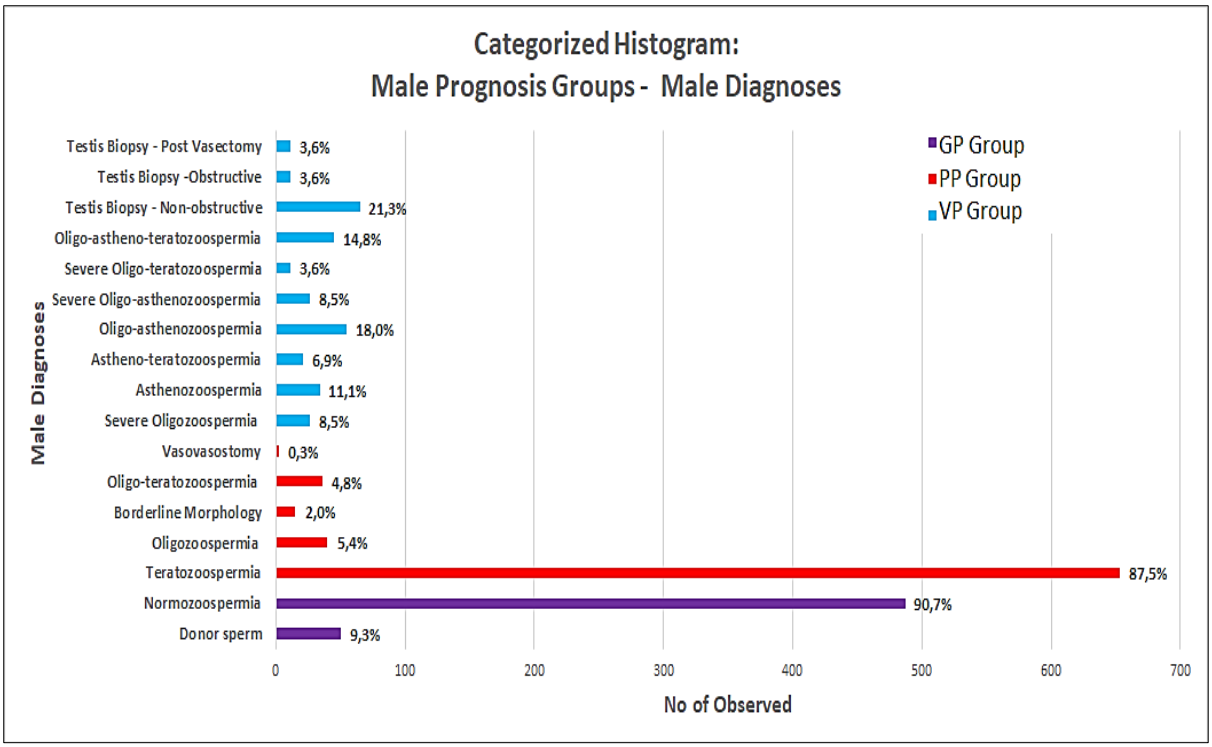

Figure 3.1. A categorized histogram depicting the distribution of male diagnoses within each male prognosis group.

The most frequent female diagnosis, from which oocytes were retrieved across all three male prognosis groups was Idiopathic / No female factor indicated, comprising of 30.2% in the GP group, 46.8% in the PP group and 48.9% in the VP group (*see Table 3.2*).

The remainder of the primary female oocyte diagnoses are further described in Table 3.2 and Figure 3.2.

Table 3.2. *Descriptive table depicting with frequencies of oocyte records and percentages of all female oocyte diagnoses, in each male prognosis group, which had normal (2PN) fertilization and available syngamy timings.*

Male Prognosis Groups	**GP Group**		**PP Group**		**VP Group**	
Primary Female Oocyte Diagnosis	**N (*oocyte records*)**	**%**	**N (*oocyte records*)**	**%**	**N (*oocyte records*)**	**%**
Advanced Maternal Age	91	16.9	182	24.4	59	19.3
Anovulation	6	1.1	9	1.2	0	-
Donor Oocytes	145	27.0	55	7.4	42	13.8
Endometriosis	14	2.6	13	1.7	2	0.7
Idiopathic / No female factor indicated	162	30.2	349	46.8	149	48.9
Oligomenorrhea	3	0.6	0	-	0	-
PCO/PCOS	30	5.6	78	10.5	7	2.3
Poor Ovarian Reserve	10	1.9	16	2.1	25	8.2
Premature Ovarian Failure	6	1.1	18	2.4	1	0.3
Recurrent Miscarriage	6	1.1	2	0.3	17	5.6
Same-sex couples / Single Female	0	-	6	0.8	0	-
Tubal Factor	64	11.9	18	2.4	3	1.0
Totals	**537**		**746**		**305**	

The distribution of zygote female diagnoses between the male prognosis groups were significantly different ($P < 0.01$), as depicted in Figure 3.2.

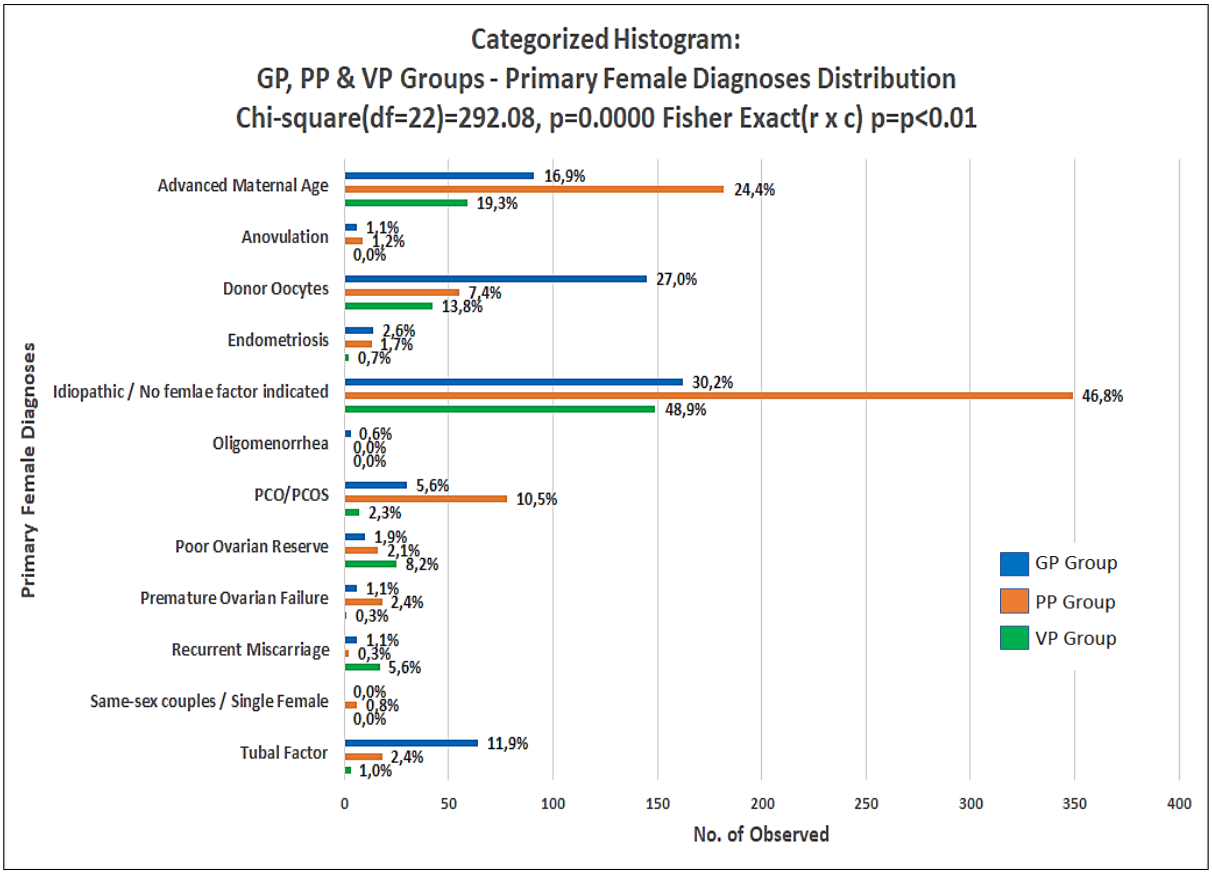

Figure 3.2. A categorized histogram depicting the distribution of primary female diagnoses of each zygote record within and between the three male prognosis group i.e. GP, VP and PP.

A two-way summary table of the chi-square analysis of the female diagnoses between the male prognosis groups is available in Appendix D.

The mean female patient age per treatment cycle in the GP group was 34.7 ± 6.31 years, the PP group age was 35.73 ± 5.16 years and the VP group age equal to 34.05 ± 5.65 years. The GP consisted of 112, the PP group of 172 and the VP group of 74 treatment cycles (*see Table 3.3*).

Table 3.3. Descriptive table of number of patient cycles, mean female patient age (years) per treatment cycle, and standard deviation overall and of each male prognosis group.

Group	*N (Patient cycles)*	*Mean Female Patient Age (years)*	*Age Standard Deviation*
GP	112	34.70	6.31
PP	172	35.73	5.16
VP	74	34.05	5.65
Total	358	35.06	5.67

There was no significant difference in the mean average female age per treatment cycle between the male prognosis groups ($P = 0.12$) (*see Fig 3.3*). Post hoc analysis results showed a significant difference in mean female patient age in the PP group compared to the VP ($P = 0.03$). Mean female patient age per treatment cycle was not significantly different between the GP and PP ($P = 0.13$) groups or between the GP and VP groups ($P = 0.45$).

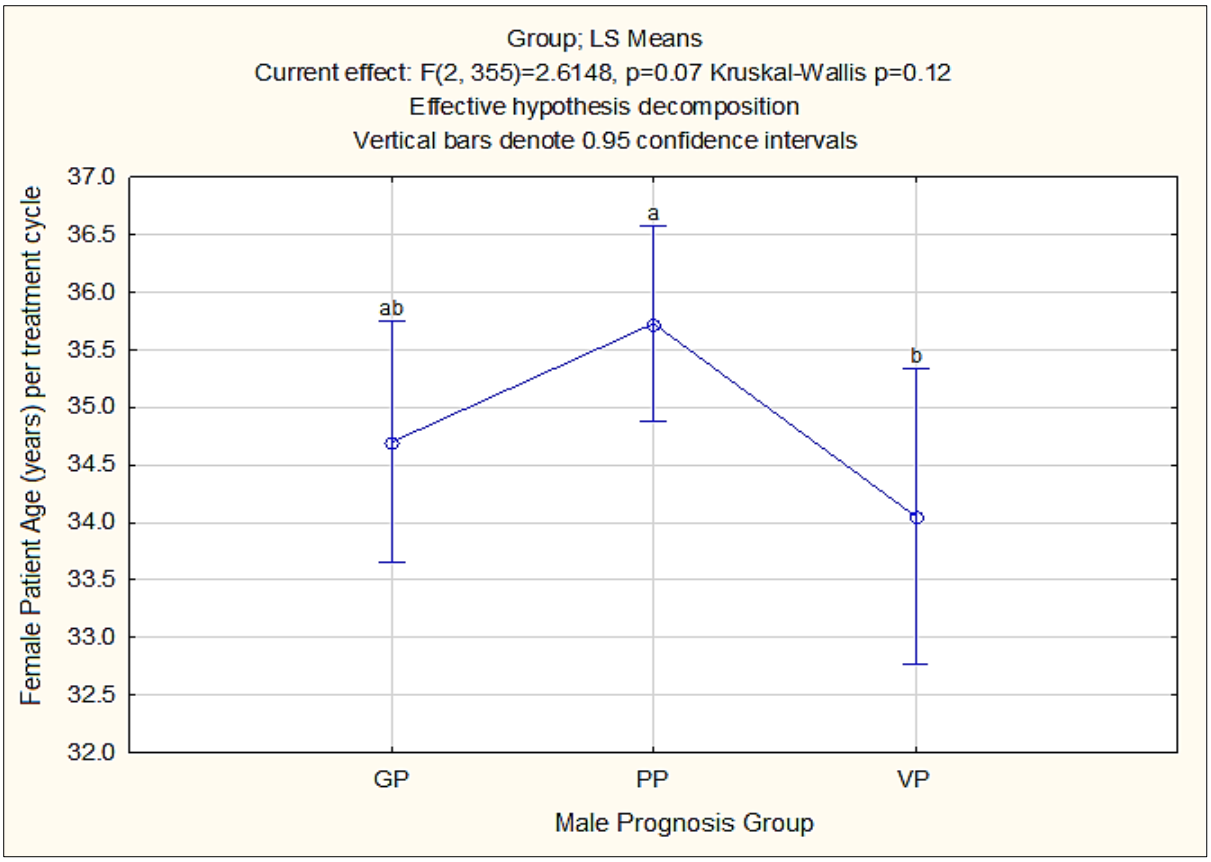

Figure 3.3. A Least-squares means (LS means) plot depicting the average (mean) female patient age (years) per treatment cycle in the three male prognosis groups (a vs b = $P < 0.05$).

2. Fertilization

In total, the database consisted of 3103 oocyte records before undergoing filtering (*see Table 3.4*). In the GP group (n = 1043 records) there were 416 abnormally or non-fertilized oocytes and 627 normally fertilized oocytes. The normal fertilization rate for the GP group was 60.12%. In the PP group (n = 1443 records) 594 oocytes fertilized abnormally or not at all and 849 oocytes fertilized normally. The normal fertilization rate in the PP group was 58.84%. In the VP group (n = 617 records) there were 282 abnormally or non-fertilized oocytes and 335 normally fertilized oocytes. The normal fertilization rate in the VP group was 54.29%. The total normal fertilization rate for all three groups combined was equal to 58.36% (1811/3103).

Table 3.4. Summary of the number of normally and abnormally fertilized oocytes, sample sizes and fertilization rates overall and of each male prognosis group.

Groups	Failed or Abnormally Fertilized Oocytes	Normally Fertilized Oocytes	Totals	Fertilization Rate
GP	416	627	1043	60.12%
PP	594	849	1443	58.84%
VP	282	335	617	54.29%
Totals	1292	1811	3103	58.36%

**Fertilization rate (%): number of normally fertilized oocytes divided by the total number of oocytes inseminated.*

There was no significant difference in the normal fertilization rate between the male prognosis groups (GP, PP, VP) (*P* = 0.19) (*see Figure 3.4*). Post-hoc test results showed no significant differences in normal fertilization rate between the GP and PP groups (*P* = 0.75); the GP and VP groups (*P* = 0.14) or the PP and VP groups (*P* = 0.08). Although it seemed that the VP group had a lower fertilization rate compared to the other groups, the result did not reach statistical significance. Therefore, all three groups had the same chance for normal fertilization.

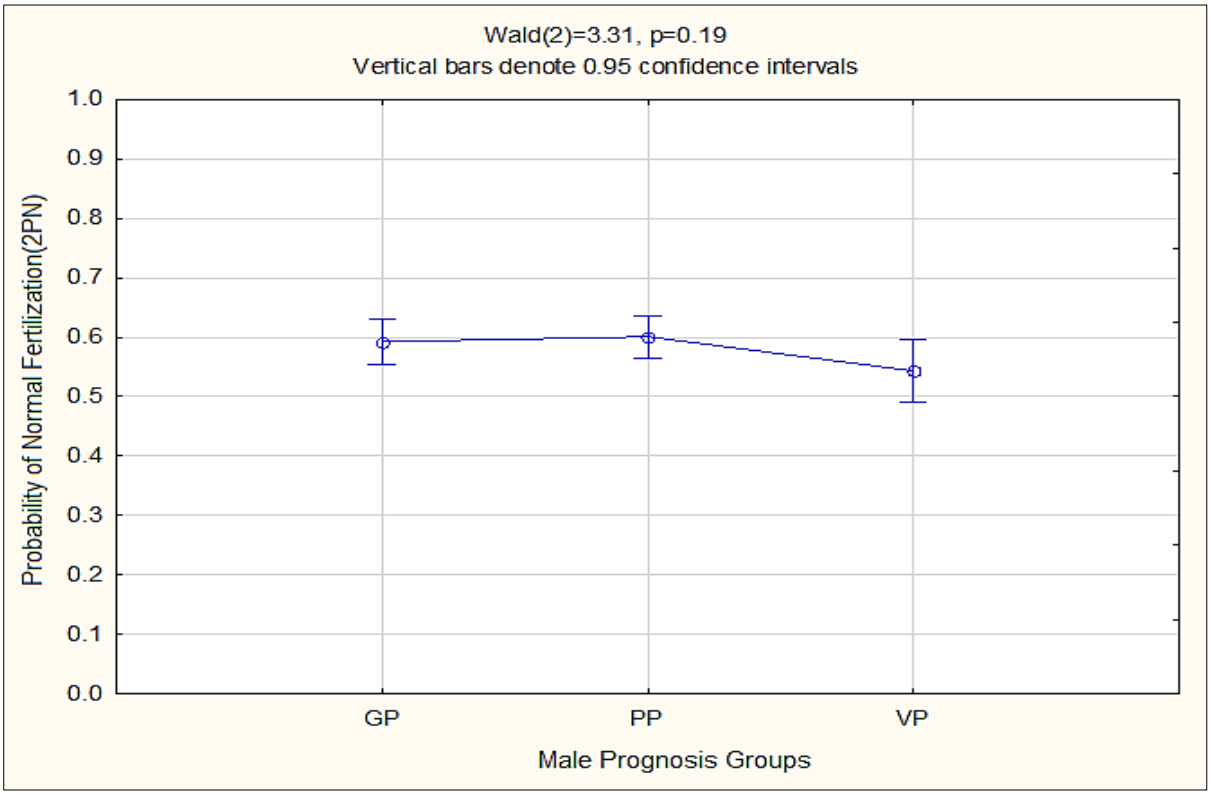

Figure 3.4. Graph depicting the GEE results of the probability of each male prognosis group (GP, PP, VP) to successfully fertilize an oocyte normally (2PN).

Neither female age (*P* = 0.52) nor male prognosis (*P* = 0.19) showed any effect on fertilization rate (*P* = 0.19) (*see Table 3.5*).

Table 3.5. Table depicting the Wald parametric test results, showing the effect of female age and male prognosis group on the chances of normal fertilization.

Effect	Df	Wald	*P*. value
Age	1	0.40	0.52
Group	2	3.31	0.19

3. Syngamy timing (hours)

Different statistical tests were used to analyse the effect of male prognosis group on syngamy timing.

The overall median syngamy timing was 19.60 hrs, with a mean of 20.16 hrs and standard deviation (sd) of 4.1 hrs. The minimum equalled 0.3 hrs and maximum 55.4 hrs. The 25th percentile was at 17.5 hrs and 75th percentile at 22.1 hrs (*see Figure 3.5*).

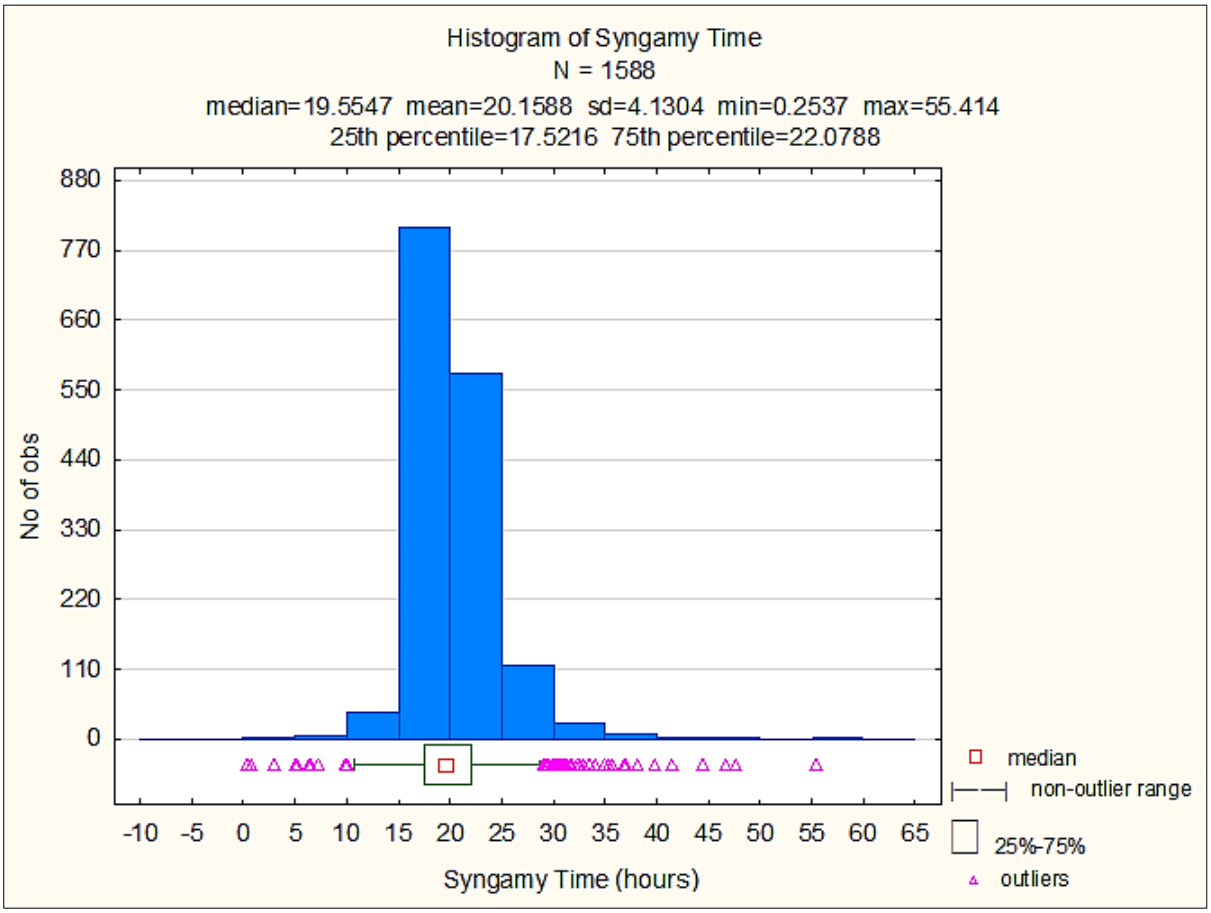

Figure 3.5. Histogram depicting frequency of observed syngamy timings (hrs) across all records, median, mean, minimum, maximum and outliers.

The GP group had a mean syngamy timing of 19.73 hrs ± 3.69 hrs. The PP group had a mean syngamy timing of 20.21 hrs ± 4.14 hrs. The VP group had a mean syngamy timing of 20.80 hrs ± 4.71 hrs. The overall mean syngamy timing of the three male prognosis groups were 20.16 ± 4.13 hrs (*see Table 3.6*).

Table 3.6. Table showing descriptive statistics of the mean syngamy timings (hrs) and standard deviations overall and of each male prognosis group.

Groups	N	Mean Syngamy Timing	Standard Deviations
GP	537	19.73	3.69
PP	746	20.21	4.14
VP	305	20.80	4.71
Totals	1558	20.16	4.13

The mean difference in syngamy timing between the GP and PP groups were 0.47 hrs (*P* = 0.23); between the GP and VP groups were 1.06 hrs (*P* = 0.02) and between the PP and VP groups were 0.58 hrs (*P* = 0.16). Covariates such as female age and male prognosis group were accounted for. The only significant difference in syngamy timings were between the GP and VP groups (*P* = 0.02). Patients in the VP group therefore had a significantly longer syngamy timing in comparison to the GP group (*see Table 3.7*).

Table 3.7. Least Significant Difference test outcome of the comparison between the three male prognosis groups with regards to syngamy timings(hrs)

Group 1	Group 2	Mean Syngamy Timing Difference	Standard Error	*P*. value
GP	**PP**	-0.47	0.39	0.23
GP	**VP**	-1.06	0.46	0.02
PP	**VP**	-0.58	0.42	0.16

The overall *P*-value calculated was equal to 0.07, showing no significant difference between male prognosis groups. Post hoc analysis showed a significant difference between the syngamy timings of the GP and VP groups (*P* = 0.02) (*see Figure 3.6*).

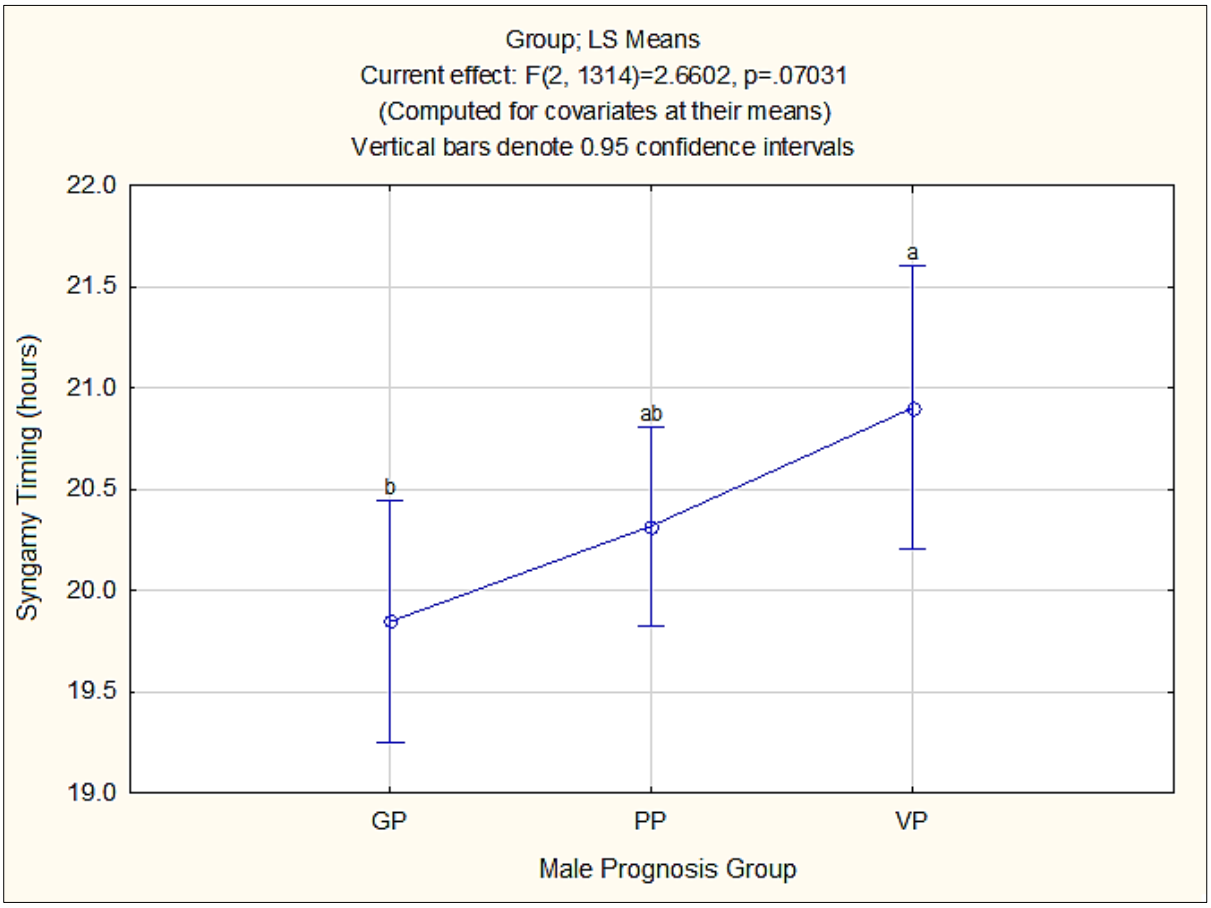

Figure 3.6. A Least-squares means (LS means) plot depicting the mean syngamy timings and the 95% confidence intervals for all three male prognosis groups (a vs b = *P* < 0.05).

The confounding effects of female age and male prognosis group on the syngamy timings were tested and showed that female age had a significant effect on syngamy timing (*P* = 0.02). Overall male prognosis group, had no significant effect on syngamy timing (*P* = 0.07) (*see Table 3.8*).

Table 3.8. Summary of the fixed effect test result of female age and different male diagnosis groups on syngamy timing.

Effect	*Num. DF	*Den. DF	*F	*P*. value
Age	1	1314	5.63	0.02
Group	2	1314	2.66	0.07

*(*Num. DF = Degrees of freedom of the numerator, Den DF = Degrees of freedom of the denominator, F = Variation Between Sample Means / Variation Within the Samples.)*

4. Blastulation

The total number of blastocysts for all three groups were equal to n = 1209. The overall blastulation rate was 66.67% (1209/1811). In the GP group, 406 embryos reached initiation of blastulation, with a blastulation rate of 64.75% (406/627). The PP group had 591 embryos that reached initiation of blastocyst stage with a blastulation rate of 69.61% (591/849), while the VP group had 212 embryos that reached initiation of blastocyst stage with a blastulation rate of 63.28% (212/335) (*see Table 3.9*).

Table 3.9. Summary of the number of normally fertilized oocytes which either failed to reach blastocyst stage or successfully reach blastocyst stage. Blastulation rates of male prognosis groups and overall rate is shown.

Group	Blastulation Failure	Blastulation Reached	Totals	Blastulation Rate
GP	221	406	627	64.75%
PP	258	591	849	69.61%
VP	123	212	335	63.28%
Totals	602	1209	1811	66.76%

The GEE results for the probability of normally fertilized oocytes –in each male prognosis group – to successfully reach blastulation had a *P*-value calculated equal to 0.25. Therefore, male prognosis group had no significant effect on blastulation rate overall. The post-hoc test also showed no significant differences in reaching blastulation between the three male prognosis groups (*see Figure 3.7*).

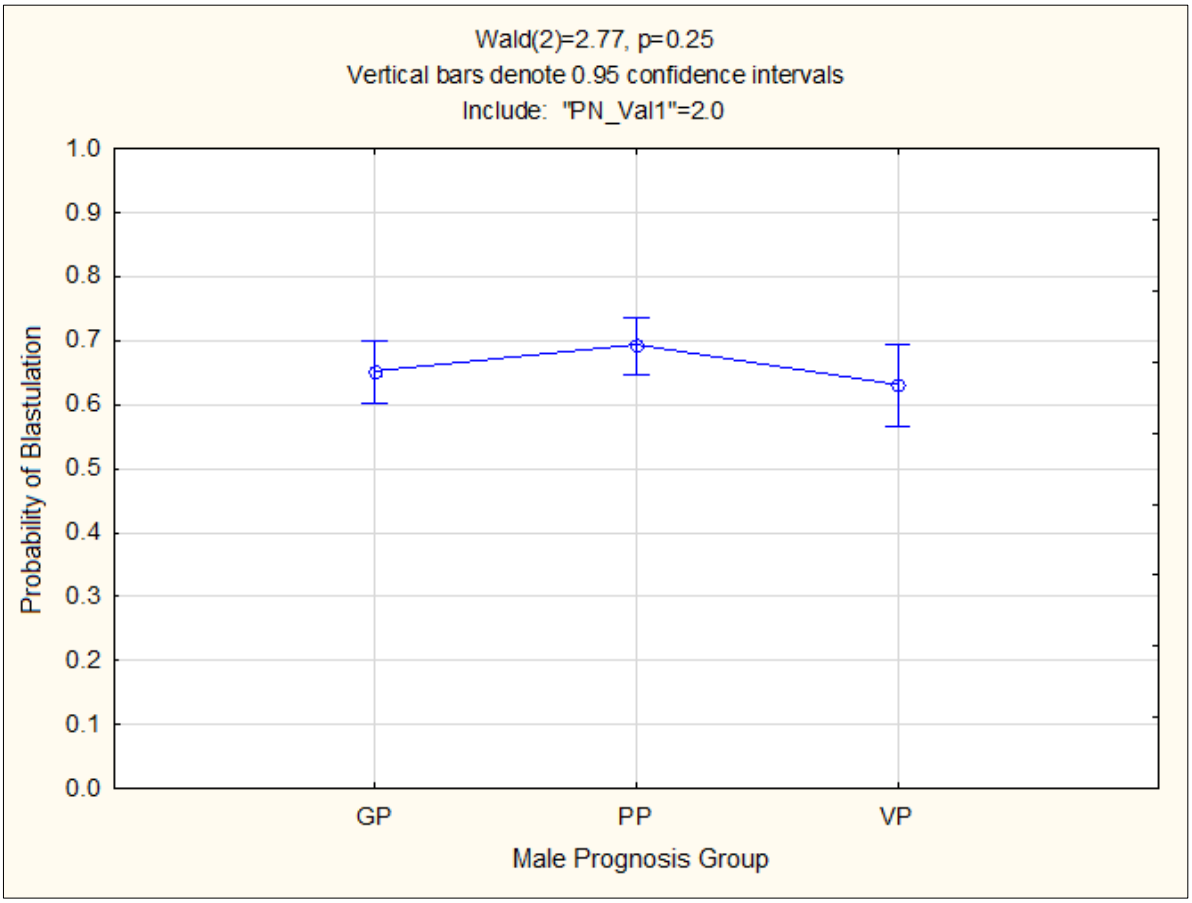

Figure 3.7. Graph depicting the GEE outcome for the probability of normally fertilized oocyte to reach blastulation in each male prognosis group.

Two factors, female age and male prognosis group and their confounding effects on blastulation were tested using the fixed effect test. Female age was shown to have a significant effect on blastulation rate ($P < 0.01$). However, male prognosis group showed no significant effect on blastulation rate ($P = 0.25$) (*see Table 3.10*).

Table 3.10. Summary of the Wald parametric test of female age and different male diagnosis groups on blastulation rate.

Effect	Degrees of freedom	Wald	*P*. value
Age	1	8.05	0.00
Group	2	2.77	0.25

5. Effect of syngamy timing on blastocyst grading

5.1 Blastocyst expansion and syngamy timing

Blastocyst expansion was significantly associated with shorter syngamy timing as the overall *P*-value was significant ($P < 0.01$) (*see Figure 3.8*).

Post-hoc analysis showed significant differences between specific blastocyst expansion grades and syngamy timing.

Early blastocysts (EB) showed a significantly shorter syngamy timing compared to compact morulae (CM) ($P = 0.04$), but showed no difference compared to grade 1 expanded blastocysts. Grade 1 expanded blastocysts showed similar syngamy timings in comparison to grade 2 or grade 3 expansions. However, grade 1 expanded blastocysts did show shorter syngamy timing compared to CM ($P = 0.02$). Syngamy timing of grade 2 expanded blastocysts significantly differed from CM ($P < 0.01$), EB ($P = 0.01$) and grade 4 expansion ($P = 0.02$). Grade 2, grade 3 and grade 5 expansions showed similar syngamy timings. Grade 3 blastocyst expansion showed significantly shorter syngamy timing compared to CM ($P < 0.01$), EB ($P < 0.01$) and grade 4 expansion ($P < 0.01$), but did not show any difference compared to grade 5 expansion. Grade 4 blastocyst expansion had significantly shorter syngamy timings compared to most other blastocyst expansion grades: CM ($P < 0.01$), EB ($P < 0.01$), grade 1 ($P < 0.01$), grade 2 ($P = 0.02$), and grade 3 ($P < 0.01$). There was no difference in syngamy timing between grade 4 and grade 5 expansions. Grade 5 blastocyst expansion showed significantly shorter syngamy timing compared to CM ($P < 0.01$), EB ($P = 0.01$) and grade 1 ($P = 0.05$). The complete LSD test table of expansion and syngamy timing is available in Appendix E.

Covariates such as female age, male prognosis group and the inter-effect between grade and group were included in the analysis. The post hoc results for female age showed no significant effect on blastocyst expansion ($P = 0.16$). The inter-effect between grade of expansion and male prognosis group also showed no significant effect ($P = 0.71$).

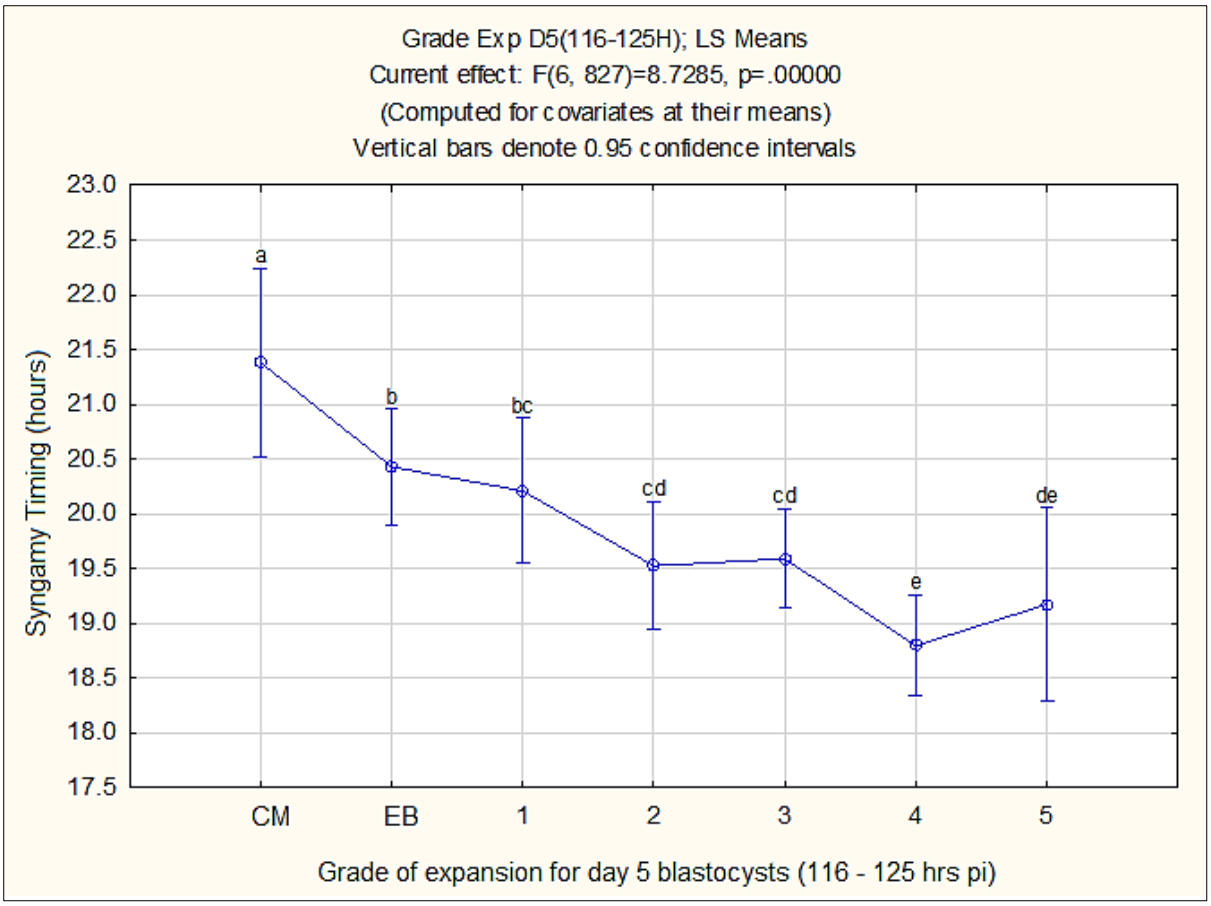

Figure 3.8. A Least-squares means (LS means) plot showing overall syngamy timing versus the degree of blastocyst expansion on day 5 (a vs. b vs. c vs. d vs. e = $P < 0.05$).

The better grade of blastocyst expansion was associated with shorter syngamy timings in all three male prognosis groups (*see Figure 3.9*). Results showed no significant difference between the three groups with regard to blastocyst expansion and syngamy timings ($P = 0.80$). The post-hoc tests showed no significant difference between the GP and PP groups ($P = 0.09$), also not between GP and VP groups ($P = 0.62$) and neither between the PP and VP groups ($P = 0.52$).

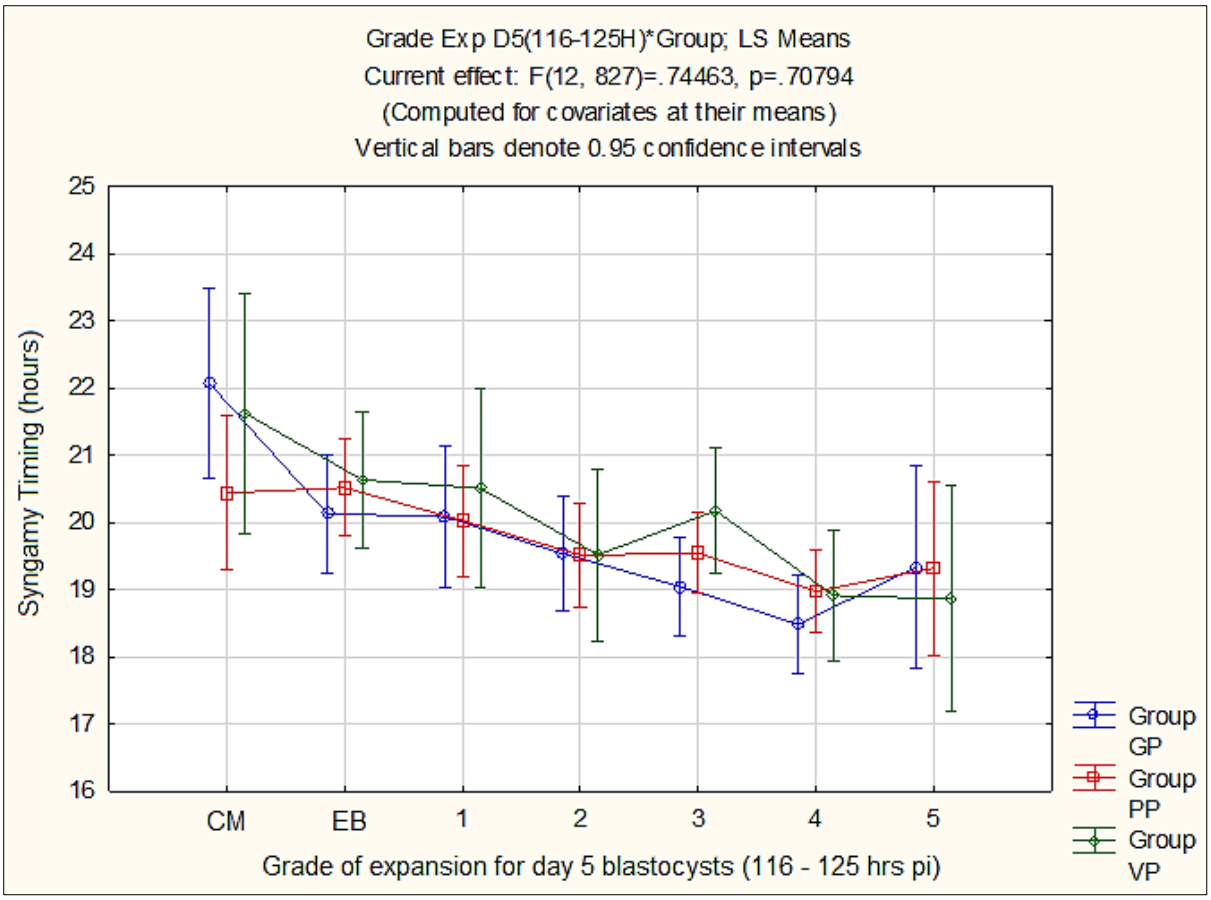

Figure 3.9. A Least-squares means (LS means) plot showing syngamy timings(hrs) versus day 5 blastocyst expansion grade for all three male prognosis groups.

5.2 Blastocyst ICM grading and syngamy timing

There was no significant overall effect of syngamy timings on blastocyst ICM grade on day 5 of embryo culture (P = 0.36) (*see Figure 3.10*). Post hoc analysis also indicated no significant syngamy timing effect between ICM A and B grading (P = 0.22); ICM A and C grading (P = 0.26) and ICM B and C grade (P = 0.09). Female age and male prognosis groups also had no significant effect (P = 0.19 and P = 0.62, respectively). The inter-effect between male prognosis groups and grade of ICM was also not significant (P = 0.15).

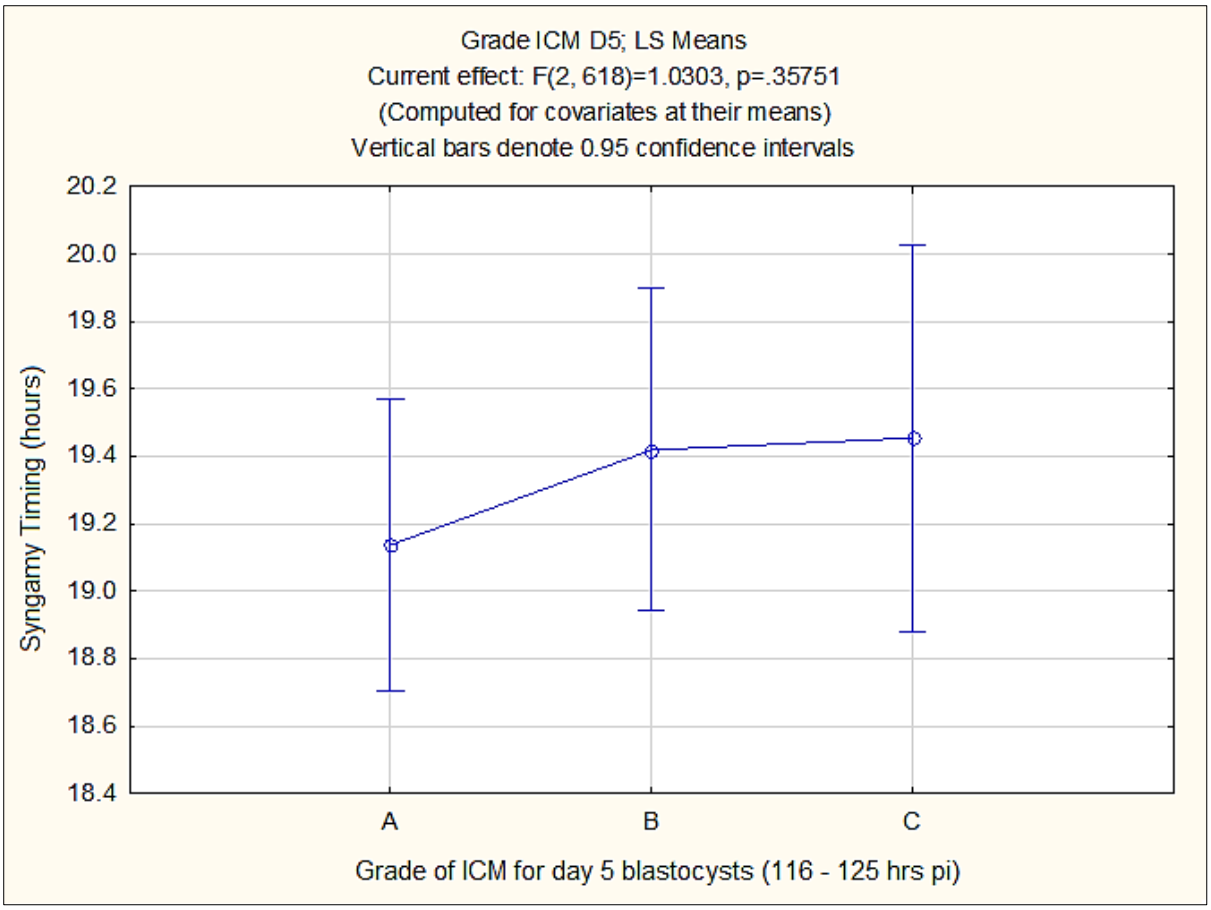

Figure 3.10. A Least-squares means (LS means) plot of overall syngamy timing (hrs) versus Inner Cell Mass (ICM) grade of day 5 of blastocyst.

There was no significant overall effect of syngamy timings on ICM grade of day 5 blastocysts between the three male prognosis groups ($P = 0.62$) (*see Figure 3.11*). All post hoc analysis results were also not significant ($P \geq 0.06$) for: Grade A ICM in the GP group in comparison to grade C ICM in the GP group ($P = 0.06$); grade A ICM in the GP group compared to grade B ICM in the VP group ($P = 0.06$) and grade A ICM in the GP group compared to grade A ICM in the PP group ($P = 0.07$).

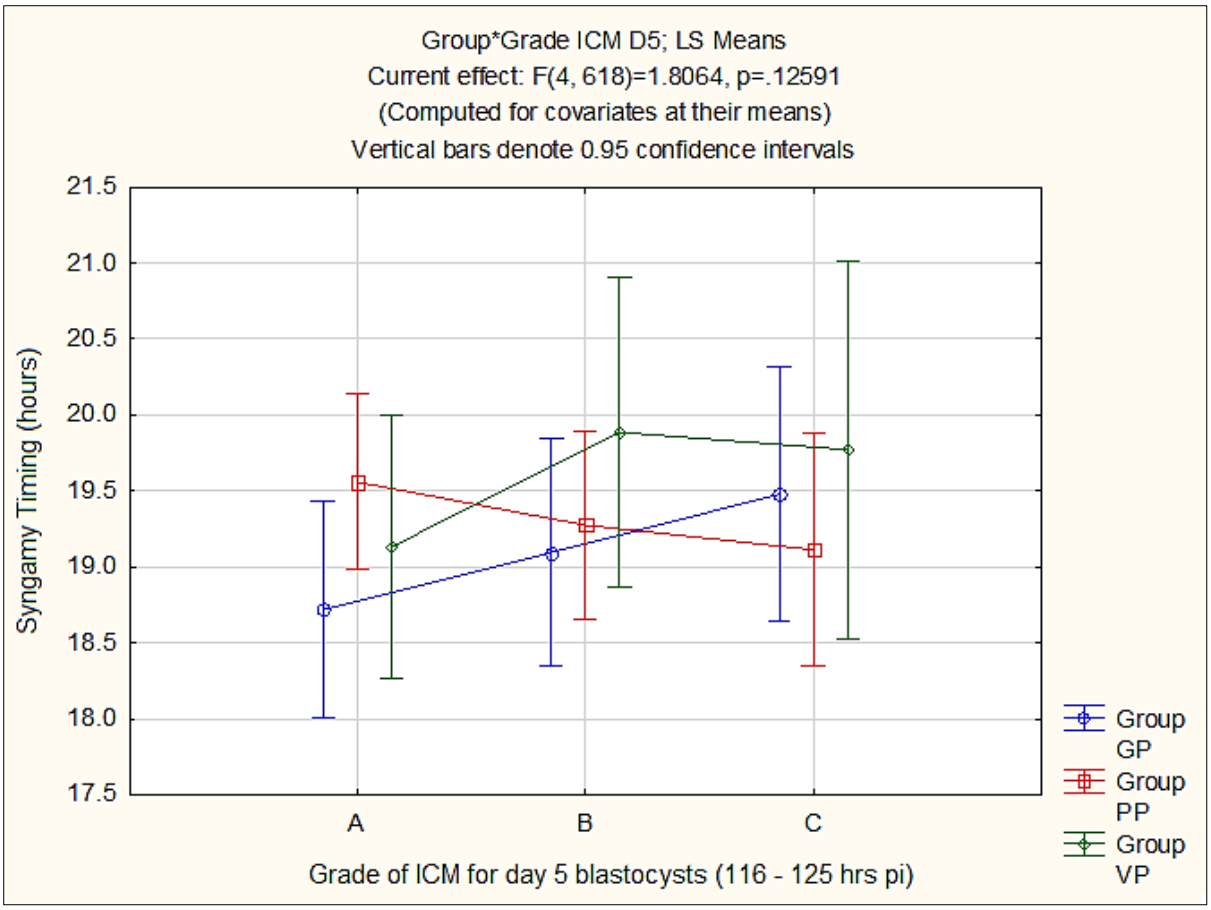

Figure 3.11. A Least-squares means (LS means) plot showing syngamy timings(hrs) versus Inner Cell Mass (ICM) grading of day 5 blastocysts for all three male prognosis groups.

5.3 Blastocyst TE grading and syngamy timing

There was no significant overall effect of syngamy timings on blastocyst trophectoderm grade on day 5 of embryo culture (P = 0.07) (*see Figure 3.12*). However, post hoc analysis indicated a significant syngamy timing effect between blastocyst trophectoderm A and B grades (P = 0.02). Grade A blastocyst trophectoderm had a significantly shorter syngamy timing compared to grade B blastocyst trophectoderm gradings. However, post hoc analysis showed no significant effect of syngamy timing on grade B versus grade C trophectoderm (P = 0.37) or grade A trophectoderm versus grade C trophectoderm (P = 0.26) blastocyst gradings.

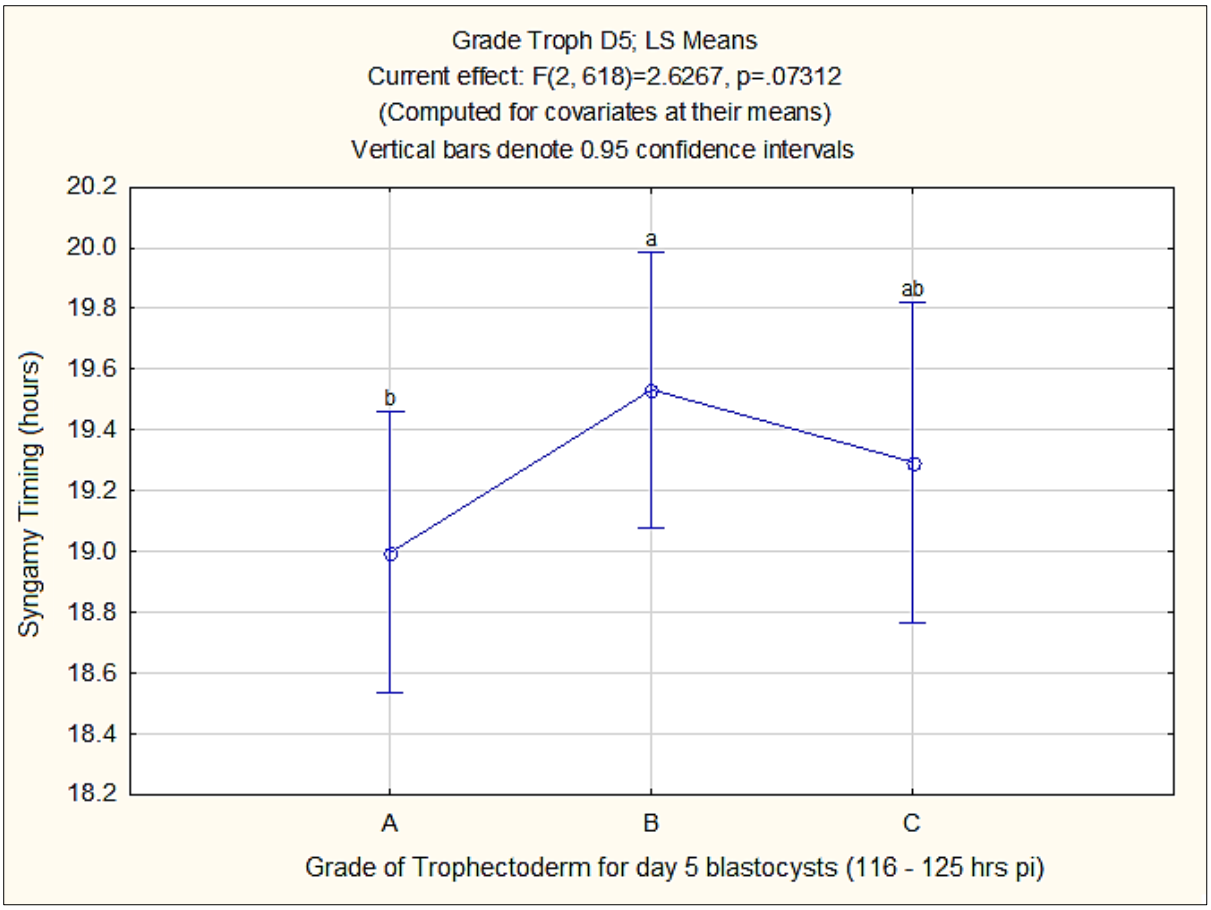

Figure 3.12. A Least-square means (LS means) plot showing overall syngamy timing(hrs) versus day 5 blastocyst trophectoderm grade. (a vs b = $P < 0.05$).

Although syngamy timing seemed to be shorter in blastocysts with better trophectoderm grading, the result did not reach statistical significance. There was no significant overall effect of syngamy timings on day 5 blastocyst trophectoderm grades or between the three male prognosis groups ($P = 0.31$) (*see Figure 3.13*). However, post hoc analysis results showed a significantly shorter syngamy timing in the GP group with A grade trophectoderm grading versus the VP group with B grade trophectoderm grading ($P = 0.05$). In the VP group, Grade A trophectoderm blastocysts also had a significantly shorter syngamy timing compared to grade B trophectoderm blastocysts ($P = 0.04$).

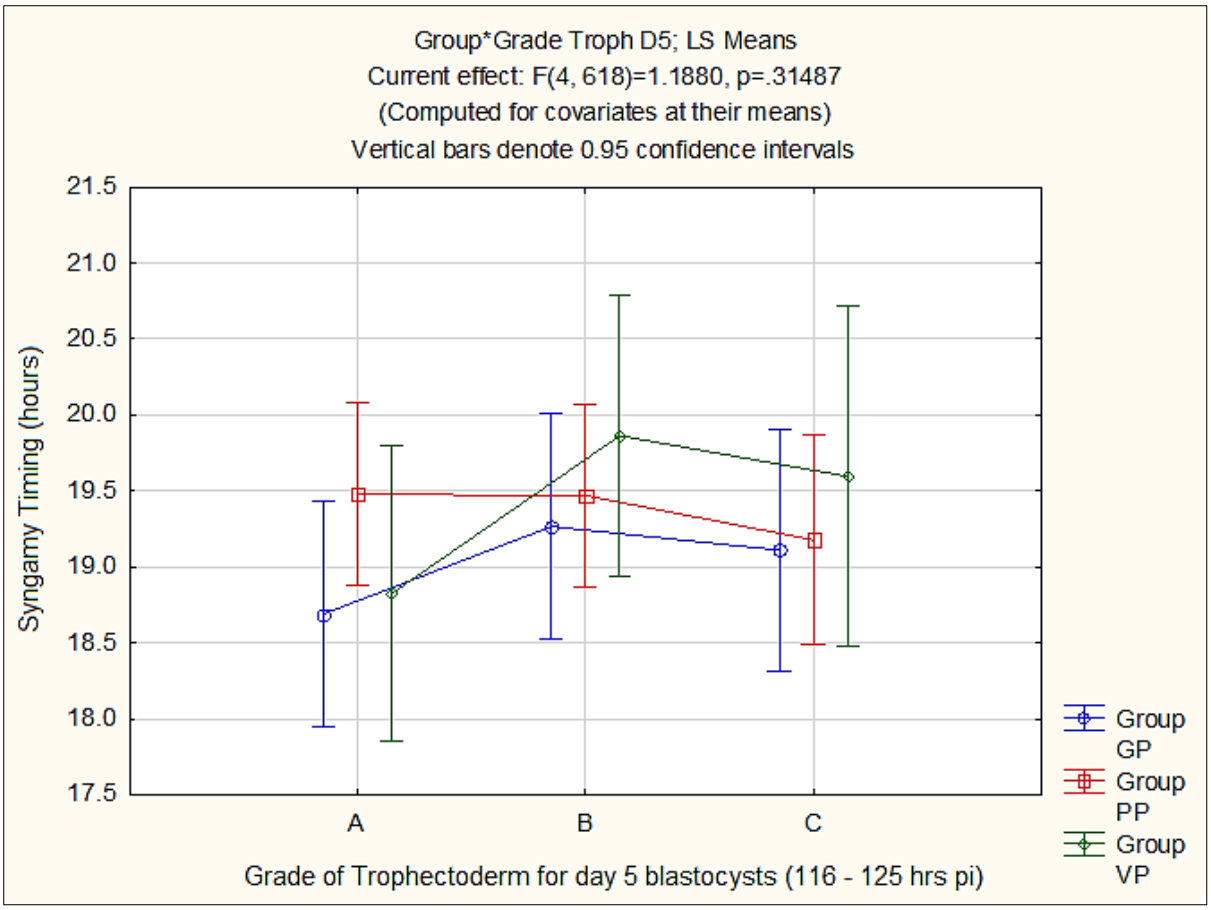

Figure 3.13. A Least-squares means (LS means) plot showing syngamy timings versus day 5 blastocyst trophectoderm grading for all three male prognosis groups.

CHAPTER 4

DISCUSSION

ART success and treatment outcome is highly dependent on embryo selection for embryo transfer, especially in SET laboratory settings. Research is ongoing to identify strong predictive markers, specifically non-invasive, observable and/or measurable embryo developmental markers, for implantation and ultimately successful live birth.

Human embryo culture in CO_2 incubators has advanced to being equipped with TL technology and software. This enables embryologists to study and incorporate many different embryo developmental traits during an uninterrupted and undisturbed embryo culture environment for blastocyst quality prediction and embryo selection. The predictive value of many of these embryo morphokinetic characteristics is still controversial, as the repeatability is limited in different laboratory settings, besides from large trials (Meseguer *et al.*, 2012, Basile *et al.*, 2014b). The effects of patient diagnoses and other factors on these events have also not yet been established and large studies like that of Meseguer *et al.* (2012) included mainly oocyte donation patient populations.

In this TL study, one of the early fertilization developmental events, **syngamy**, was specifically investigated in relation to **male factor prognosis**. Sperm quality and syngamy are most probably highly interconnected due to the contribution of the functional spermatozoan centrosome (Wirka *et al.*, 2014) and also the sperm DNA quality affecting EGA (Castillo *et al.*, 2018) and thus **blastulation** (Desai *et al.*, 2009).

The time duration to syngamy, as well as the effect of male factor infertility on syngamy timing were investigated to elucidate the possible paternal effect/contribution of the functional centrosome.

Our study also aimed to investigate subsequent advanced embryo developmental traits during blastulation, in relation to initial syngamy time and male prognosis. In advanced stages of embryo development, the spermatozoon plays a significant role during EGA and contributes to blastulation. Therefore, the effect of syngamy time on day 5 embryo development was specifically investigated by evaluating the **blastocyst quality** with regards to blastocoel **expansion size**, **inner cell mass quality** and **trophectoderm quality**. The effect of the three different male prognosis groups on day 5 embryo development (blastocyst stage) was also investigated.

1. Descriptive Data

The distribution of normally fertilized zygotes across **female diagnoses** between the three male prognosis groups were significantly different in this study. The main female diagnosis in all three male prognosis groups and overall, was idiopathic. Idiopathic diagnosis was defined according to the primary female diagnosis of each oocyte record, regardless of the male diagnosis result. The high prevalence of idiopathic female diagnoses for the recorded and analysed oocyte data in the GP male prognosis group (30.2%) agrees with literature stating that 8% to 40% of female patients has idiopathic diagnoses (Ray *et al.*, 2012, Gelbaya *et al.*, 2014, Abdelazim *et al.*, 2018).

However, the PP and VP male prognosis groups had a higher prevalence of idiopathic oocyte records (46.8% and 48.9%). This could be attributed to the fact that these two male prognosis groups had a definitive male infertility factor indication, which was not included as a primary female oocyte diagnosis, but rather indicated as idiopathic diagnosis. Therefore, these records were automatically categorized into the idiopathic infertility diagnosis, increasing the number of oocyte records.

The GP group also had a relatively high prevalence of donor oocytes, which indicates that there were female infertility factors in this group that played a role and increased donor oocyte ART treatment in this group.

The female study population was very heterogeneous amongst and across male prognosis groups and was one of the study limitations.

The second highest prevalence in both the PP and VP groups was advanced maternal age, a factor well known to have an impact on ART according to the literature (Lawler *et al.*, 2007, Liu *et al.*, 2011). Female age between the three male prognosis groups was calculated and analysed per treatment cycle, which showed no overall significant difference. This minimized the possible confounding effect that female oocyte age could have had on the results. However, there was an increased female age in the PP group in comparison to the VP group, which could be expected when taking into account that the PP group's second highest female oocyte diagnosis was advanced maternal age. Female age was compensated for in this study as a covariate by statistical analysis.

The primary outcome of this study was to investigate the **three male prognosis groups**, thus female diagnosis was not compensated for. Study population size would have been reduced dramatically if only donor oocytes were included and therefore all female diagnoses were included, also giving a more realistic representation of the populations that clinics observe every day.

The male diagnoses were intentionally categorized according to prognosis groups and therefore it was expected that the diagnosis distribution of the three male prognosis groups would be very different. The different male diagnoses within the poor prognosis groups were not evenly distributed, where teratozoospermia was the major diagnosis in the PP group (87%), and non-obstructive azoospermia (21%) and oligo-asthenozoospermia (18%) was the majority in the VP group.

2. Fertilization

The overall **fertilization rate** (58.36%) of the three male prognosis groups was not statistically significant. Therefore, all three male prognosis groups (GP: 60.12%; PP: 58.84% and VP: 54.29%) had the same chance to normally fertilize an oocyte.

This agrees with a study by Yerebasmaz *et al.* (2017) who showed no significant difference in the fertilization rate of a male factor study group (50.8%) in comparison to the control group (51.2%). Borges Jr *et al.* (2017) also showed no significant difference in normal fertilization rates between male factor (75.72 ± 20.50%) and tubal factor (78.42 ± 18.36%) groups. Lammers *et al.* (2015) reported no significant difference in fertilization rate between the origin of spermatozoa samples in ejaculated spermatozoa (61.00%) compared to surgically removed spermatozoa (48.48%).

Post hoc analysis results showed there was no significant difference between the fertilization rates of the GP and PP male prognosis groups or the GP and VP male prognosis groups. The fertilization rate was slightly higher for the GP group compared to the PP and VP groups. The PP group had a slightly lower fertilization rate compared to the GP group and the VP group. The VP group had the lowest fertilization rate compared to both the GP and PP groups and the difference between the VP and PP group showed a significant difference at the 90% confidence interval.

This finding of an observable decreasing trend in fertilization rate from the GP to PP to VP groups, although not statistically significant, was also reported in other studies in literature where poorer male diagnoses had lower fertilization rates. Hourvitz *et al.* (1998) showed a lower fertilization rate in non-obstructive azoospermia or oligo-astheno-teratozoospermia (42%), compared to the control group of ejaculated spermatozoa (55.5%). Verza Jr and Esteves (2008) showed a lower fertilization rate in triple defect spermatozoa (63.4 ± 25.9%) and testicular retrieved sperm from non-obstructive azoospermia patients (52.2 ± 29.3%) in comparison to other diagnoses (~73%). Another study by Zheng *et al.* (2016) also reported lower fertilization ability as the motility of severe oligozoospermia patients declined in male groups.

ICSI is the method of choice applied for treatment of poor male prognosis. The overall similar fertilization rate results across male prognosis groups in the current study could be explained based on the fact that ICSI selects the best possible sperm cell based on motility and morphology, although not necessarily selecting for normal genetic and epigenetic spermatozoa profiles (Denomme *et al.*, 2018). ICSI could therefore possibly compensate for poor male prognosis to a certain extent. The similar fertilization rate trend observed in the current study for the different male prognosis groups, could therefore be expected.

The possible confounding factor, female age, showed no statistical significant effect on normal fertilization rate. This indicates that all three male prognosis groups had the same probability for normal fertilization and female age did not affect normal fertilization chances. Normal fertilization rates were similar irrespective of male prognosis group or female age.

This finding agrees with other studies in literature. Grøndahl *et al.* (2017) showed that female age had no effect on the normal fertilization rate of zygotes, but that patients with advanced maternal age had an increased abnormally fertilized zygotes in IVF and ICSI cycles. Borges Jr *et al.* (2017) showed in women older than 35 years, the normal fertilization rates between the male factor (73.98 ± 22.33%) and tubal factor (77.83 ± 18.40%) groups had no significant difference. They also reported no significant difference in normal fertilization rates between male factor (76.41 ± 19.71%) and tubal factor (78.78 ± 18.41%) groups in women younger than 35.

In this study we included different insemination methods: IVF, ICSI and variations of ICSI insemination methods (PICSI and IMSI).

Literature reports on ART outcomes in different insemination methods are very controversial. Plachot *et al.* (2002) reported no significant difference in the fertilization rate, embryo morphology or development, pregnancy and implantation rates between ICSI and IVF patients with male factor infertility. Yoeli *et al.* (2008) showed higher fertilization rates in ICSI than in IVF (67.1% vs 43.6%), but no significant difference in the embryo quality. A meta-analysis by Johnson *et al.* (2013) showed that ICSI had better fertilization rates and a smaller risk for total fertilization failure compared to conventional IVF in couples with unexplained infertility.

In this study, ICSI was mostly indicated and applied in male factor infertility cases or in patients where female factors such as advanced maternal age was indicated as a treatment decision. The inclusion of all insemination methods is stated as a limitation of the study in the methods section.

3. Syngamy Timing

Syngamy timing and the calculation thereof are defined differently in reports in literature, with no consensus on how syngamy should be determined and/or defined. Many studies used time of insemination as the reference point (t0), which does not allow an accurate and consistent starting point, as each oocyte insemination time is not annotated individually on the TL system, but rather recorded as an overall average insemination of all oocytes (Aguilar *et al.*, 2014, Wirka *et al.*, 2014).

Studies focussing on differences in kinetic parameters between ICSI and IVF also show conflicting results. Time points delineating S-phase initiation between IVF and ICSI was shown to possibly differ in non-human models (Hewitson *et al.*, 2000). Fishel *et al.* (2018) reported that IVF embryos had a longer tPNf of 1.95 hours post insemination compared to ICSI, which was one of their secondary findings. In contrast, Cruz *et al.* (2013), found that the kinetic time points do not differ between IVF and ICSI when a pre-determined, reference time point (t0) was used. Ueda *et al.* (2012) reported that syngamy time for IVF was 21.2 ± 3.6 hours and was 20.8 ± 3.6 hours for ICSI, which was not significant.

In this study, t0 was based on time of second polar body extrusion, which is the end of meiosis and oocyte activation, as well as the start of the new cell cycle for DNA replication and events preceding syngamy. Therefore, this study's initial t0 time point allows an accurate and fixed annotation event from which genetic and fertilization events leading up to syngamy, can be investigated. This also minimizes time differences created prior to tPB2 by the method of insemination (IVF and ICSI) (Cruz *et al.*, 2013). The current study's calculation of syngamy timing is therefore the same as in the study by Ueda *et al.* (2012). Based on the fact that this study focussed on kinetic parameters to determine syngamy timing, it was decided that there was enough motivation to include both IVF and ICSI cycles.

A total of 1811 normally fertilized oocyte records were included in the study. **Syngamy timings** included for statistical analysis were recorded in 1588 cases (zygotes). The dropout of 223 records could be because some annotations could not be assessed due to overnight IVF culture, as well as poor visualization preventing imaging of the second polar body extrusion and/or the pronuclear fading. The mean syngamy timing across all three male prognosis groups was 20.16 ± 4.13 hours.

This agrees with most mean syngamy timings reported in literature. In a study by Ueda *et al.* (2012), good quality embryos had a significantly shorter syngamy time of 20.0 ± 3.3 hours, in comparison to poor quality embryos (22.10 ± 4.3 hours). They also showed that abnormally fertilized embryos had longer syngamy times of 25.10 ± 6.3 hours, which showed a significant delay compared to normally fertilized embryos. In contrast, Azzarello *et al.* (2012) later reported that PN fading associated with live birth outcome (24.52 ± 0.35 hours) and earlier PN fading with no live birth (23.10 ± 0.23 hours), but their calculation was from post insemination and not second polar body extrusion. Their main outcome measured was live birth rate, which is different from this current study's outcome of blastocyst grading parameters, therefore it is difficult to directly compare syngamy timing results.

One study included syngamy (Wirka *et al.* 2014). More studies reported on morphokinetic parameters and **embryo quality** on day 2 and 3, also without day 5 (Lemmen *et al.*, 2008, Wirka *et al.*, 2014).

Lemmen *et al.* (2008) used TL to identify markers after normal fertilization, which could be correlated to good quality embryos on day 2 and also implantation. Time-points included were PN fading after fertilization, nuclei appearance and disappearance in blastomeres and synchronization of cell cleavages. The results showed positive correlations between earlier PN disappearance after fertilization, earlier first cell cytokinesis and increased blastomere number on day 2 of embryo development. Positive correlations with pregnancy rate was found when nuclei appeared synchronously after first cell cytokinesis and good quality embryos were associated with synchronous development, but time range references were not reported.

Wirka *et al.* (2014) reported that only 30% of the abnormal syngamy embryos resulted in good or fair quality embryos in comparison to the normal syngamy group where 60% resulted in good or fair quality embryos.

Several studies have reported on the influence of morphokinetic parameters on live birth and implantation (Meseguer *et al.*, 2011, Azzarello *et al.*, 2012).

Meseguer *et al.* (2011) reported a time duration of 4.04 hours from the time of PN fading until the time of first cell cleavage, which delineates the G_2 and M phases. Results showed that the most predictive parameters of possible **implantation** were time of division to 5 blastomeres, time between division to 3 blastomeres and to 4 blastomeres and duration of the two-cell cycle. Azzarello *et al.* (2012) investigated whether PN morphology and PN fading could predict **live birth** outcome. They found that embryos with PN fading earlier than 20.45 hours post insemination did not result in live birth.

In the current study there was no overall statistical significance between the **syngamy timings** of the three **male prognosis groups**, although there was a noticeable trend showing that poorer male prognosis resulted in longer syngamy timings. Specifically, the VP group had 1.06 hours longer syngamy timing compared to the GP group, which was significant. Although not significant, the PP group had 0.47 hours longer syngamy timing compared to the GP group. Therefore, results in the current study suggest that male prognosis does affect the timing of syngamy.

The effect of male prognosis on syngamy timing has not been studied extensively, but literature suggests that male factor infertility could play a role in embryo morphokinetic events (Wirka *et al.*, 2014, Denomme *et al.*, 2018).

Denomme *et al.* (2018) reported that there was an increased miscarriage rate in OAT patients compared to normal male diagnosis patients. The methylation and transcriptome of OAT male factor patients resulted in aberrations which translate in epigenetic events that consequently lead to reduced reproductive potential of the embryo's associated with poor male sperm (Denomme *et al.*, 2018). The observed trend in our study of longer syngamy timing, increasing in poorer male prognosis groups, could be linked with study outcomes in literature.

The significant effect of **female age** on **syngamy timing** has been reported in literature. In a prospective, benchtop incubator study by (Lawler *et al.*, 2007), it was shown that zygotes from females younger than 36 years entered syngamy earlier and had a better implantation potential when entering syngamy before 23 to 24 hours post insemination. Therefore, female age could have had a significant effect on syngamy timing, where younger patients (zygotes) could result in a significantly shorter syngamy timing. In the current study, female age was thus taken into account as a confounding factor and compensated for in statistical analysis of the study.

4. Blastulation

The overall **blastulation rate** of the three male prognosis groups in the current study was 66.76% and the post-hoc tests for blastulation rate between the three male prognosis groups showed no statistical significance. This indicates that in all three male prognosis groups the chance for reaching blastulation (tSB) was similar.

The blastulation rate found in this study is in accordance with blastocyst developmental rates (competency rate of ≥ 40% and benchmark rate of ≥ 60%) reported in the Vienna consensus expert meeting (Apter *et al.*, 2017). Regarding blastulation in the different male prognosis groups in the current study, the slightly higher (5-6%) non-significant blastulation rate results seen for the PP group, compared to the two others, is difficult to explain. A possible explanation could be because female factor infertility was less prevalent in the PP group compared to that in the VP and GP group. It is also possible that the increased blastulation rate seen in the PP group could be because ICSI compensated for poor semen parameters, especially sperm morphology. Therefore, adverse effects might be avoided when embryologists select the spermatozoa with the best morphology, which might not be representative of the entire sample (French *et al.*, 2010). French *et al.* (2010) speculated that ICSI compensates for poorer quality samples by fertilizing oocytes with microscopically selected spermatozoa with the closest resemblance to normal morphology, which might make the pre-treatment morphology assessment irrelevant. This could also be true for the VP group, which had a blastulation rate similar to the GP group. However, this argument is challenged by other findings that state that IVF and ICSI show no significant difference in blastocyst rate (Westphal *et al.*, 2003).

In this study, although there was a significant difference seen in the syngamy timing between the GP and VP group, there was no significant difference seen in blastulation rate. This could be because the spermatozoa contribute the centrosome during syngamy, which is a separate functional entity to the DNA. After syngamy and before blastulation, the embryo genome is activated, where the spermatozoa DNA quality plays a role. Literature has shown that male diagnosis groups, where spermatozoa were retrieved from the testis (VP), might have less DNA damage in comparison to ejaculated spermatozoa (GP) (Bungum *et al.*, 2004, Weissman *et al.*, 2008, Moskovtsev *et al.*, 2010, Esteves *et al.*, 2015). In addition to the fact of less DNA damage, ICSI also compensates for poor male prognosis and could therefore be the explanation to why these results were seen in this study.

In the literature, there is contradictory evidence concerning blastulation rate in different male diagnosis groups. Some studies show no significant difference between male spermatozoa source or group, where others do. Desai *et al.* (2009) reported no difference in compaction or blastulation rates, respectively, amongst epididymal (74%, 38%), obstructive azoospermic testicular (69%, 42%) or non-obstructive azoospermic testicular (79%, 38%) spermatozoa. French *et al.* (2010) also did not observe any difference in blastulation rate (41 to 50%) amongst different groups. Their results showed that fertilization, implantation, pregnancy and live birth rates were not statistically different between morphological spermatozoa groups and no effect was observed for blastocyst development or quality. Lammers *et al.* (2015) showed no significant difference in blastulation rates between ejaculated (52.52%) and surgically retrieved spermatozoa (59.07%).

Other studies however, reported differences in blastulation rates from different spermatozoa sources or diagnosis groups (Mazzilli *et al.*, 2017, Bartolacci *et al.*, 2018). Mazzilli *et al.* (2017) showed a significant difference in blastulation rate per fertilized oocyte in moderate male factor (48.6%) and non-obstructive azoospermia (40.6%) in comparison to normozoospermia (49.3%). Results by Bartolacci *et al.* (2018) showed a significant difference in blastulation rates, from the poorest male group (concentration < $1x10^6$ per ml): 50.0 (33.3–66.3%) compared to the better male group (concentration ≥ $15x10^6$ per ml): 55.6 (40.0–75.0%). However, they did not find a difference in development of top-quality blastocysts between the male groups.

Although in this current study the possible effect of **syngamy timing** on **blastulation rate** was not specifically investigated, it should be mentioned that there are studies suggesting a correlation. Wirka *et al.* (2014) showed significantly lower blastulation rates in embryos with atypical phenotypes (21.5%) i.e. abnormal syngamy, abnormal first cytokinesis, abnormal cleavage and chaotic cleavage in comparison to normal syngamy embryos (44.9%).

Several studies have been performed on morphokinetic parameters to predict **blastulation**. One study included syngamy, and the effect on outcomes (Wong *et al.*, 2010, Desai *et al.*, 2014, Milewski *et al.*, 2015).

Wong *et al.* (2010) identified three cleaving morphokinetic data parameters which could accurately predict blastocyst formation before day 4 of embryo development, using an algorithm they developed. The kinetic parameters included: first cell cytokinesis duration (t2-tPNf), time between the first mitosis completion and second mitosis initiation (t3-t2) and the time duration between the second and third mitoses (t4-t3).

Milewski *et al.* (2015) also created a predictive model based on early cleaving morphokinetic parameters for blastocyst development on day 5. Parameters investigated included the first (t2), second (t3), third (t4) and fourth (t5) blastomere divisions, as well as the time duration between these divisions. The first (t2), fourth (t5) as well as time between the second (t3) third (t4) division was used as predictive timings for blastocyst formation. The study by Desai *et al.* (2014), reported on time to syngamy in poor quality and non-blastulating embryos (26.8 ± 8.3 hours) compared to blastocysts frozen (25.2 ± 3.0 hours). They also showed that tPNf, t2, t4, t8, s1, s2, s3 and cc2 were significantly different in embryos forming blastocysts in comparison to embryos either failing to reach blastulation or forming poor quality blastocysts. Literature suggests that **blastulation rate** is also not influenced by **insemination method**, therefore supporting the inclusion of all insemination methods in this study. Westphal *et al.* (2003) showed no difference between ICSI and IVF with regards to blastulation (78% in IVF and 73% in ICSI) or viable pregnancy rates (51.4% in IVF and 55% in ICSI) in patients who had abnormal semen parameters or previously failed IVF cycles.

Literature showed different findings on the effect that **maternal age** has on **blastulation**, with some studies showing earlier blastulation in younger patients than older patients, and other studies showing no influence of maternal age on blastulation rates (Sepúlveda *et al.*, 2011, Hickman *et al.*, 2013). In our study, female age did have a significant effect on the probability of blastulation, but was compensated for in all statistical analysis.

5. Syngamy timing and blastocyst grading parameters

In the current study, **syngamy timing** of an embryo showed a significant overall correlation with regards to the degree of **blastocyst expansion.** The post hoc analysis results revealed that there were specific significant differences in syngamy timing between day 5 blastocyst expansion grades. Shorter syngamy timings were significantly associated with advanced blastocyst development. Blastocyst expansion grades 1, 3, 4 and 5 had shorter syngamy timings compared to CM embryos. Blastocyst expansion grades 2, 3, and 5 had shorter syngamy timings compared to EB embryos. Expansion grades 4 and 5 had shorter syngamy timings compared to grade 1 of blastocyst expansion, grade 4 compared to grade 2 of blastocyst expansion, and grade 4 compared to grade 3 of blastocyst expansion. This result was the most significant and important outcome of the study.

Studies that correlate blastocyst expansion and syngamy specifically are limited. Most studies only correlate blastocyst expansion with implantation and/or live birth. Blastocyst expansion has been shown in literature to be a very important predictive factor of LBR (Hill *et al.*, 2013, Thompson *et al.*, 2013, Desai *et al.*, 2016, Du *et al.*, 2016).

Hill *et al.* (2013) reported that implantation rates were significantly higher in expanded (64%) and hatched blastocysts (63%) compared to morula (38%) and early blastocysts (44%). Live birth rate was significantly decreased in morula (13%) compared to early blastocysts (37%), expanded blastocysts (53%) and hatched blastocysts (58%). They subsequently also reported that blastocyst stage development, including TE quality was significantly associated with implantation and live birth.

Thompson *et al.* (2013) found similar values for LBR outcomes associated with blastocyst stage, with expanded blastocysts (49.5%), hatching blastocysts (50%) and early blastocysts (36.7%). Results by Desai *et al.* (2016) showed that day 5 blastocyst expansion grades 3 and 4 was predictive of successful outcomes in comparison to day 6 blastocysts with the same expansion grade. Delayed blastocyst expansion, such as grades 1 and 2 in comparison to more expanded grades 3 and 4, negatively impacted implantation. Blastocyst expansion was therefore a strong predictor of outcomes such as implantation, clinical pregnancy and live birth rates. They also showed that univariate analysis of ICM and TE grading correlated positively with outcomes.

Du *et al.* (2016) conducted a similar study to Desai *et al.* (2016), investigating the effect of all three parameters of blastocyst grading on live birth. Results also showed the degree of blastocoel expansion was predictive of live birth in fresh and vitrified-warmed single embryo transfer. Expansion degrees of 1 and 2 had lower birth rates in comparison to degrees 3 and 4. Neither ICM nor TE was correlated with live birth using logistic regression analysis.

The **syngamy timings** of the three different **male prognosis groups** regarding blastocyst **expansion** showed no significant difference. This might indicate that the male prognosis played a lesser role in the degree of blastocyst expansion with regards to syngamy timing. Post hoc analysis also showed no significant difference between the syngamy timings for each expansion grade between the three male prognosis groups.

This agreed with a study by Bartolacci *et al.* (2018) who investigated the effects of different spermatozoa diagnoses on overall embryo quality. They reported no significant difference in top quality blastocyst formation rate between men who had different degrees of oligozoospermia ($<1 \times 10^6$ per ml, $<5 \times 10^6$ per ml, and $<15 \times 10^6$ per ml) compared to the control group ($\geq 15 \times 10^6$ per ml). However, this study did not investigate individual blastocyst grading parameters, but only overall grading.

In light of this study's findings, which showed significant correlations of syngamy timing with degree of blastocyst expansion, irrespective of male prognosis, it could be argued that syngamy may be an important developmental event for expanding potential. Syngamy timing could therefore be a selection marker of advanced blastocyst expansion, which in turn, according to literature, is an important indicator of implantation potential.

The overall **syngamy timing** of day 5 **inner cell mass (ICM)** grading showed no significant correlation. The post hoc analysis between the day 5 ICM gradings according to syngamy timings, also showed no statistical significance. Although the post hoc results showed no significant difference between syngamy timing of different ICM gradings, shorter syngamy timings were associated with better quality ICM grade (A-grade shorter than B-grade). The syngamy timings started to plateau between B- and C-grade ICM blastocysts. Female age as a possible confounding factor, showed no significant influence on the grade of ICM.

The overall **syngamy timing** of all three **male prognosis groups** and the effect on day 5 **inner cell mass (ICM)** grading showed no significant correlation. Post hoc analysis also showed no statistically significant difference between male prognosis groups when correlation between day 5 ICM gradings and syngamy timings in each group were tested. However, there was an observable trend in the GP group, with poorer ICM grading associated with longer syngamy timing. The VP group showed a similar result, but the PP group showed an opposite trend, with a poorer ICM grading associated with shorter syngamy timing. The reason for this finding is difficult to explain and needs further investigation with larger sample sizes. This might underline the less important role of the ICM in blastocyst grading due to lower correlations with implantation potential.

Recent studies in the literature investigated the role of the ICM and whether it has predictive value for outcomes such as live birth, however studies are contradictory. The findings of this study showed that ICM was neither influenced by syngamy timing, nor male prognosis group, which is similar to literature previously mentioned (Bartolacci *et al.*, 2018) that reported no difference in top blastocyst formation rate between male diagnosis groups.

In the current study, **syngamy timing** compared to **day 5 trophectoderm grade** had no overall statistical significance, but was significant at the 90% confidence interval. The post hoc analysis revealed a statistical significance between the syngamy timings of A-grade and B-grade trophectoderm qualities. A grade trophectoderm had a significantly shorter syngamy timing compared to B grade trophectoderm. Syngamy timing between B- and C-gradings, as well as A- and C-gradings showed no significant differences. It is difficult to explain this non-significant result. Since the epithelial layer is not necessarily uniform and TE can be assessed on different focal planes, leading to possible uncertainty and inconsistent grading. These discrepancies could be more frequent between B- and C-gradings, which might be more difficult to differentiate, whereas A grade TE could be more discernible. However, the final grading is mostly corroborated by more than one embryologist. Therefore, a larger sample size could possibly clarify this unexpected result.

The comparison of syngamy timings with day 5 trophectoderm grading across all three male prognosis groups was not statistically significant. However, the post hoc analysis showed a significantly shorter syngamy timing between an A-grade TE of the GP group and a B-grade TE of the VP group, as well as between the A- and B-grade trophectoderms in the VP group itself. Although not statistically significant, longer syngamy timing seemed to lead to poorer trophectoderm gradings in the GP and VP groups. The PP group did not follow a similar trend, with a consistent syngamy timing for all three trophectoderm gradings. The reason for this observation is difficult to explain. One possibility is due to the categorization of the PP group, which included borderline morphology diagnoses and oligozoospermia diagnosis (irrespective of morphology). Patients with a borderline morphology or low sperm count with increased normal morphology, possibly resulted in a better spermatozoa population for ICSI sperm selection.

There are no published studies investigating syngamy timing of different male prognoses and its effect on day 5 blastocyst quality grading. However, the effect of semen parameters on other morphokinetic events and subsequent implantation and live birth rates have been evaluated, but also not on the quality of individual parameters of day 5 blastocysts (French *et al.*, 2010, Lammers *et al.*, 2015, Desai *et al.*, 2018).

French *et al.* (2010) studied the effect of male sperm morphology on blastocyst quality and surprisingly observed a higher percentage of good quality blastocysts in the most severely teratozoospermic group with 0% normal forms in comparison to patients with ≥5% normal forms (37% vs 28%). They attributed this outcome to a possible lower female factor infertility prevalence in this group.

Lammers *et al.* (2015) compared the effect of fresh ejaculated and surgically (testicular and epididymal) retrieved spermatozoa in ICSI on embryo morphokinetic parameters. Clinical outcomes were measured and they showed that the outcomes were comparable between the groups of different sperm sources. Although, some early morphokinetic parameters between the groups had significant differences, time points included t3, t8 and s2. They concluded that there was no difference in morphokinetic analysis between fresh ejaculated and surgically retrieved sperm, but stated that more studies are required on the relationship between sperm origin and late morphokinetic parameters such as blastocyst development.

Desai *et al.* (2018) also investigated kinetic embryo development and clinical outcomes in different spermatozoa sources, i.e. epididymal, testicular (obstructive and non-obstructive) and ejaculated normal spermatozoa. A lower compacting embryo rate in non-obstructive testicular spermatozoa compared to the others were seen, which is indicative of paternal activation (Desai *et al.*, 2009). In the testicular sourced spermatozoa, significantly lower percentage of embryos showed kinetics typically attributed to high quality embryos. Results showed no difference in CPR between the groups and a non-significant decline in implantation and birth rate of testicular spermatozoa compared to epididymal spermatozoa and the control group.

The syngamy timing results in this current study, could predict TE quality between grade A and B. In conjunction with findings in the literature our results suggest that syngamy timing could possibly be an early predictor of TE quality and ART outcomes, such as implantation and live birth rates. Several studies have reported on blastocyst grading and pregnancy outcomes, not taking morphokinetic information into account. Some studies reported no correlation between ICM and live birth (Ahlström *et al.*, 2011, Hill *et al.*, 2013, Ebner *et al.*, 2016). Other studies showed that TE is predictive of implantation and other outcomes such as live birth (Ahlström *et al.*, 2011, Hill *et al.*, 2013, Ebner *et al.*, 2016) and some only showed correlation of TE when a univariate analysis was performed (Desai *et al.*, 2016).

The study by Ahlström *et al.* (2011) found that all three parameters (Expansion, TE and ICM) of blastocyst grading had an effect on live birth, but when confounding factors were adjusted for, only TE showed a strong correlation with live birth. The results of their GEE models showed a significant incremental increase in live birth rate from poor to better TE gradings: grade C (8.0%), compared to B (33.9%) and compared to A (49.9%).

Hill *et al.* (2013) reported that implantation and live birth rates were higher in TE grades A (67%, 57%, respectively) compared to B (51%, 40%, respectively). Implantation rates were significantly lower in grade C (25%) compared to grade A TE, and live birth rates also lower in grade C TE, but was not statistically significant. However, they reported no significant difference in implantation or live birth rates between different ICM gradings A (63%, 53%, respectively), B (58%, 52%, respectively) or C (0%, 0%, respectively).

Ebner *et al.* (2016) reported that quality and cell number of the TE outweighed ICM with regards to live birth outcome prediction, whereas other studies, as discussed above, reported a correlation between ICM and live birth in the case of a univariate analysis (Desai *et al.*, 2016, Du *et al.*, 2016).

Thompson *et al.* (2013) reported an association with TE morphology and LBR. LBR between the three TE scoring groups were 50% in the good scored group, 41.9% in the fair scored group and 30% in the poor scored group. No association was seen between LBR and ICM morphology. Thompson *et al.* (2013) reported on the combined outcome of blastocyst expansion and TE morphology in LBR. Results were better in the hatching blastocyst group (52% with good TE score, 51.1% with fair TE score, 43.7% with poor TE score), in comparison to expanded blastocysts (46.5% with good TE score, 42.7% with fair TE score and 25.9% with poor TE score), with LBR being the lowest in the early blastocyst group (31.4% with good TE score, 32.9% with fair TE score and 38.7% with poor TE score).

Syngamy timing and individual day 5 blastocyst quality grading parameters have not been studied or extensively reported in literature, although the relationship between morphokinetic time points, embryo quality, blastulation and ART outcomes have been investigated.

The study by Desai *et al.* (2014) reported on time to syngamy in blastocysts transferred (24.8 ± 2.6 hours), in blastocysts implanting (24.1 ± 2.5 hours), compared to blastocysts not implanting (26.2 ± 2.7 hours).

The results of this study, in conjunction with findings reported in the literature, suggest that syngamy timing may be a relevant early morphokinetic time point correlating with blastulation, especially blastocyst expansion and to some extent with trophectoderm morphology, irrespective of male factor prognosis.

Combined results on blastocyst expansion and TE morphology of our study is therefore strongly suggested to have an even further impact on other outcomes such as LBR. Therefore, our findings might be of significant value for future embryo selection for transfer and ART outcomes.

Since literature has shown that both our main findings, blastocyst expansion and trophectoderm, are strong predictive markers of ART outcomes, syngamy timing could be an early indicator of blastocyst expansion and trophectoderm quality, therefore also possibly predicting implantation and other associated ART outcomes.

CHAPTER 5

CONCLUSION

A limited number of studies correlating syngamy timing and individual blastocyst grading parameters, as well as the effect of male factor prognosis have been published. In our study, we aimed to elucidate the effect of male prognosis on syngamy timing and subsequent blastulation.

Our results showed that syngamy timing correlated significantly to **blastocyst expansion** overall, with shorter syngamy timing resulting in greater expansion. No statistical significance between the mean syngamy timings of the three male prognosis groups were found. However, it seemed that mean syngamy timing increased with poorer male prognosis. No statistical significance with **ICM** grading overall or between male prognosis groups were seen; although an A-grade ICM seemed to have a shorter mean syngamy timing. No statistical significance with **trophectoderm** grading overall or between male prognosis groups were found. Although, an A-grade trophectoderm showed a significantly shorter syngamy timing in comparison to B-grade trophectoderm.

The inherent process of syngamy and effect on the final embryo genome product thereof could therefore have an effect on blastulation formation and blastocyst grading outcome. Blastocyst expansion and TE quality, the two most important grading parameters for embryo selection and transfer, was associated with syngamy timing in this study and with improved ART outcomes in the literature.

ICSI used in conjunction with sperm selection, could compensate for poor male prognosis, and although not statistically significant, a definite observable trend was seen in the study - showing a longer syngamy timing in the poorer male prognosis groups. The centrosome and inherent spermatozoa quality, a final contributor to the syngamy process, could possibly have an effect on ART outcomes.

Since only blastocyst quality correlated with syngamy timing, irrespective of male prognosis group, this finding could be explained by the inherent spermatozoon DNA quality that only affects syngamy and not blastocyst quality grading.

Based on literature and in conjunction with the findings of this study, we suggest that the grade of blastocyst expansion and trophectoderm epithelium quality between A and B could be predicted as early as syngamy timing. Syngamy timing could therefore be applied either as a single or additional parameter in embryo selection methods to increase the probability of implantation and live birth.

CHAPTER 6

FUTURE PROSPECTS

Due to the limited literature on syngamy, its calculation inconsistency and lack of association with sperm quality, blastulation and ART outcomes, further research is needed.

Suggestions for the future research and improvement of the current study should take the limitations of the study into consideration.

The female population of the study was heterogenous, therefore maternal influences such as female diagnosis was not considered in the study analysis. Female oocyte quality might also play a role in syngamy and EGA. Abnormal oocyte DNA and cytoplasm contents might also be a contributing factor to aneuploid embryos or abnormal syngamy. These factors could be eliminated to some extend in future studies by including a homogenous female population of only donor oocytes or by categorizing oocytes into groups according to female diagnosis (PCOS, endometriosis, anovulation, poor responders, recurrent pregnancy loss).

DNA fragmentation tests, such as TUNEL, could be included in addition to semen analysis diagnosis to ensure better defined male prognosis groups. However, it will be challenging to perform TUNEL assays on severe oligozoospermia and testicular retrieved spermatozoa samples.

In order to determine whether the observed trends and results that showed significance at the 90% confidence intervals could reach statistical significance at the 95% confidence interval, two aspects of this study can be addressed. Stricter categorization of male prognosis groups would possibly optimize diagnosis separations and could improve delineation of differences between groups. Larger sample sizes might also elucidate whether true differences exist by increasing the statistical significance.

The predictive value of syngamy timing for blastocyst expansion and trophectoderm epithelium should be validated and confirmed through prospective studies, as this study only analysed this retrospectively.

A multicentred study would evaluate the applicability of syngamy timing as an important early morphokinetic predictive marker for broader laboratory settings.

The outcomes measured in this study could be extended to include implantation, clinical pregnancy and live birth rates. It would be interesting and of additional value to compare pregnancy outcomes with published results readily available in the literature.

CHAPTER 7

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APPENDICES

1. Appendix A: Definition lists

General definitions

Allele	"One of two or more alternate forms of a gene" (Pierce, 2012).
Blastocyst	An embryo, 5 or 6 days after fertilization, with an inner cell mass, outer layer of trophectoderm, and a fluid-filled blastocoel cavity.
Blastulation rate	The number of blastocysts formed from normally fertilized zygotes.
Centriole	"Cytoplasmic organelle consisting of microtubules; present at each pole of the spindle apparatus in animal cells" (Pierce, 2012).
Centrosome	"Structure form which the spindle apparatus develops: contains the centriole" (Pierce, 2012).
Chromosome	"Structure consisting of DNA and associated proteins that carries and transmits genetic information" (Pierce, 2012).
Crossing over	"Exchange of genetic material between homologous but non-sister chromatids" (Pierce, 2012).
Embryo quality	Grading of an embryo based on morphological characteristics, to estimate implantation potential of embryo and serve as a method of embryo selection.
Fertilization	The penetration of the ovum by the spermatozoon and fusion of genetic materials resulting in the development of a zygote.

Implantation rate	"The number of gestational sacs observed divided by the number of embryos transferred" (Zegers and Adamson, 2009).
Intracytoplasmic sperm injection (ICSI)	A procedure in which a single motile spermatozoon is injected into the oocyte cytoplasm.
Morphokinetic events	All embryo parameters or main developmental milestones which occur from fertilization to blastulation.
Syngamy	The fusion of the nuclei from the maternal and paternal gametes.
Zygote	A diploid cell resulting from the fertilization of an oocyte by a spermatozoon, which subsequently divides to form an embryo.

Female diagnosis definition list

Anovulation	"Anovulation is the failure of the ovary to release ova over a period of time generally exceeding 3 months" (Davis and Segars, 2009)
Donor Oocytes	"An ART cycle in which oocytes are collected from an egg donor for reproductive purposes or research" (Zegers-Hochschild *et al.*, 2017).
Endometriosis	"A disease characterized by the presence of endometrium-like epithelium and stroma outside the endometrium and myometrium. Intrapelvic endometriosis can be located superficially on the peritoneum (peritoneal endometriosis), can extend 5 mm or more beneath the peritoneum (deep endometriosis) or can be present as an ovarian endometriotic cyst (endometrioma)" (Zegers-Hochschild *et al.*, 2017).
Ideopathic	"Infertility in couples with apparently normal ovarian function, Fallopian tubes, uterus, cervix and pelvis and with adequate coital frequency; and apparently normal testicular function, genito-urinary anatomy and a normal ejaculate. The potential for this diagnosis is dependent upon the methodologies used and/or those methodologies available" (Zegers-Hochschild *et al.*, 2017).
Advanced Maternal Age	"Patients 39 years of age and older" (SARA, 2014).
Recurrent Miscarriage	"The spontaneous loss of two or more clinical pregnancies prior to 22 completed weeks of gestational age" (Zegers-Hochschild *et al.*, 2017).

Oligomenorrhea	"Fewer than 9 periods per year (cycle length > 38–40 days)" (Rosenfield, 2013).
Polycystic Ovarian Syndrome	"A heterogeneous condition, which requires the presence of two of the following three criteria: (1) Oligo-ovulation or anovulation; (2) Hyperandrogenism (clinical evidence of hirsutism, acne, alopecia and/or biochemical hyperandrogenemia); (3) Polycystic ovaries, as assessed by ultrasound scan with more than 24 total antral follicles (2–9 mm in size) in both ovaries" (Zegers-Hochschild *et al.*, 2017).
Polycystic Ovaries	"An ovary with at least 12 follicles measuring 2–9 mm in diameter in at least one ovary (Rotterdam criteria). PCO may be present in women with PCOS, but also in women with normal ovulatory function and normal fertility" (Zegers-Hochschild *et al.*, 2017).
Poor Ovarian Reserve	"A term generally used to indicate a reduced number and/or reduced quality of oocytes, such that the ability to reproduce is decreased" (Zegers-Hochschild *et al.*, 2017).
Poor Ovarian Response (POR)	"A condition in which fewer than four follicles and/or oocytes are developed/obtained following ovarian stimulation with the intention of obtaining more follicles and oocytes" (Zegers-Hochschild *et al.*, 2017).
Premature Ovarian Failure	"A condition characterized by hypergonadotropic hypogonadism in women younger than age 40 years (also known as premature or primary ovarian failure). It includes women with premature menopause" (Zegers-Hochschild *et al.*, 2017).
Same sex-couples / Single Female	Female couples / single couples who require donor sperm in order to undergo ART.

Tubal Factor "Tubal abnormality resulting in dysfunction of the Fallopian tube, including partial or total obstruction of one or both tubes (proximally, distally or combined), hydrosalpinx and/or peri-tubal and/or peri-ovarian adhesions affecting the normal ovum pick-up function. It usually occurs after pelvic inflammatory disease or pelvic surgery" (Zegers-Hochschild *et al.*, 2017).

Male diagnosis definition list

Azoospermia	"The absence of spermatozoa in the ejaculate" (Zegers and Adamson, 2009, WHO, 2010).
Asthenozoospermia	Poor motility and/or forward progression (< 30% motility and/or < 2 forward progression).
Donor sperm	"The process of placing laboratory processed sperm or semen from a man into the reproductive tract of a woman who is not his intimate sexual partner, for the purpose of initiating a pregnancy" (Zegers-Hochschild *et al.*, 2017).
Normozoospermia	"All semen parameters are normal (equal to or above the lower reference limits)" (WHO, 2010).
Non-obstructive Azoospermia	"Absence of spermatozoa in the ejaculate due to lack of production of mature spermatozoa" (Zegers-Hochschild *et al.*, 2017).
Obstructive Azoospermia	"Absence of spermatozoa in the ejaculate due to occlusion of the ductal system" (Zegers-Hochschild *et al.*, 2017).
Oligozoospermia	"Low concentration of spermatozoa in the ejaculate below 15×10^6 per ml" (Zegers and Adamson, 2009).
Teratozoospermia	"Reduced percentage of morphological normal spermatozoa (≤ 4%)" (Zegers and Adamson, 2009).
Testicular Biopsy	"A surgical procedure involving one or more testicular biopsies or needle aspirations to obtain sperm for use in IVF and/or ICSI" (Zegers and Adamson, 2009).

Vasectomy	"Procedure to occlude the vas deferens. It is usually carried out bilaterally in order to secure sterilization" (Zegers and Adamson, 2009).
Vasovasostomy	Surgical reversal of a vasectomy.

2. Appendix B: Wijnland Fertility Andrology reference values.

WIJNLAND
Fertility

SEMEN PARAMETERS

NORMAL VALUES

Volume:	**≥ 1.5 ml**
pH:	**≥ 7.2**
Viscosity:	**< 5 cm**
Count:	**≥ 15x10^6/ml**
Motility:	**≥ 30%**
Forward Progression:	**≥ 2**
MAR (Antibody) Test:	**< 50%**
Morphology:	**≥ 5%**
Leucocyte count:	**≤ 1x10^6/ml**

DEFINITIONS

Normozoospermia:	**All parameters within normal values** **G-pattern: Morphology ≥ 5 tot ≤ 14% (Good pattern - good chance of natural conception)** **N-pattern: Morphology ≥ 15% (Normal pattern)**
Teratozoospermia:	**P-pattern: Morphology ≤ 4% (Poor pattern - Sub-fertile, lowered chance of spontaneous fertilization of eggs, needs intervention (AI/IVF))**
Hypospermia:	**Volume < 1.5 ml**
Hyperspermia:	**Volume > 6.0 ml**
Aspermia:	**No semen (Repeat test for retrograde ejaculation)**
Polizoospermia:	**Count > 250x10^6/ml (Not clinically significant)**
Oligozoospermia:	**Count < 15x10^6/ml**
Kriptozoospermia:	**Only very few sperm seen in concentrate of whole semen sample**
Azoospermia:	**No sperm in the ejaculate, confirmed by ≥ 2 semen analyses on concentrate of whole semen sample**
Asthenozoospermia:	**Motility < 30% and/or FP < 2**
Necrozoospermia:	**All sperm cells are dead [Confirmed with a supravital stain (SVS)]**
Immunological factor:	**> 50% MAR IgG antibodies – clinically significant**
Leucocytospermia:	**> 1x10^6/ml polymorphic leucocyte concentration**

MORPHOLOGY RESULTS

Morphology slides are stained by means of Diff-Quik [DQ] staining and evaluated according to Tygerberg Strict criteria WHO guidelines. Two or more readings are performed by two scientists and the two closest readings are reported.

3. Appendix C: Example of data spreadsheet

Embryo ID	Prognosis Group	Female Diagnosis	Male Diagnosis	Female Age	PN_Val	tPB2	tPNa	tPNf	t2	tSB	Morphological Grade
0123_1	GP	Endometriosis	Normozoospermia	34.5	2	4.81	5.81	21.6	24	88.2	3AA

4. Appendix D: Two-way table of female diagnoses

Group	Marked cells have counts > 10. Chi-square(df=22)=292.08, p=0.0000 Fisher Exact(r x c) p=p<0.01												
	Primary Female Diagnosis Advanced Mat age	Primary Female Diagnosis Unexplained	Primary Female Diagnosis Donor egg	Primary Female Diagnosis PCO	Primary Female Diagnosis Tubal factor	Primary Female Diagnosis Endometriosis	Primary Female Diagnosis Premature Ovarian Failure	Primary Female Diagnosis Oligomenorrhea	Primary Female Diagnosis Anovulation	Primary Female Diagnosis POR	Primary Female Diagnosis Recurrent Miscarriage	Primary Female Diagnosis Single Female	Row Totals
GP	91	162	145	30	64	14	6	3	6	10	6	0	537
Row %	16.95%	30.17%	27.00%	5.59%	11.92%	2.61%	1.12%	0.56%	1.12%	1.86%	1.12%	0.00%	
PP	182	349	55	78	18	13	18	0	9	16	2	6	746
Row %	24.40%	46.78%	7.37%	10.46%	2.41%	1.74%	2.41%	0.00%	1.21%	2.14%	0.27%	0.80%	
VP	59	149	42	7	3	2	1	0	0	25	17	0	305
Row %	19.34%	48.85%	13.77%	2.30%	0.98%	0.66%	0.33%	0.00%	0.00%	8.20%	5.57%	0.00%	
Totals	332	660	242	115	85	29	25	3	15	51	25	6	1588

5. Appendix E: LSD test table of syngamy timing and blastocyst expansion

Comparisons Cell {#1}-{#2}	LSD test; variable Syngamy Time (Spreadsheet in Cheyenne Steyn - MSc Prof Kidd datastel Feb 2018.stw) Simultaneous confidence intervals Effect: Grade Exp D5(116-125H)				
	1st Mean	2nd Mean	Mean Differ.	Standard Error	p
{1}-{2}	1	2	0,68	0,4	0,08
{1}-{3}	1	3	0,62	0,36	0,08
{1}-{4}	1	4	1,41	0,36	0
{1}-{5}	1	5	1,04	0,53	0,05
{1}-{6}	1	VB	-0,22	0,38	0,57
{1}-{7}	1	KM	-1,17	0,52	0,02
{2}-{3}	2	3	-0,06	0,31	0,84
{2}-{4}	2	4	0,73	0,32	0,02
{2}-{5}	2	5	0,35	0,5	0,48
{2}-{6}	2	VB	-0,9	0,35	0,01
{2}-{7}	2	KM	-1,86	0,49	0
{3}-{4}	3	4	0,79	0,25	0
{3}-{5}	3	5	0,42	0,46	0,37
{3}-{6}	3	VB	-0,84	0,29	0
{3}-{7}	3	KM	-1,79	0,45	0
{4}-{5}	4	5	-0,38	0,46	0,41
{4}-{6}	4	VB	-1,63	0,29	0
{4}-{7}	4	KM	-2,59	0,46	0
{5}-{6}	5	VB	-1,25	0,48	0,01
{5}-{7}	5	KM	-2,21	0,6	0
{6}-{7}	VB	KM	-0,95	0,46	0,04

6. Appendix F: Health Research Ethics Committee Approval Letter

Health Research Ethics Committee (HREC)

Approval Notice

New Application

09/02/2018

Project ID :0751

HREC Reference # S17/08/158

Title: Effect of human male patient diagnosis on syngamy timing and blastulation rate using time-lapse technology.

Dear Miss Cheyenne Steyn,

The **New Application** received on 20/09/2017 was reviewed by members of the **Health Research Ethics Committee 2 (HREC2)** via **expedited** review procedures on 09/02/2018 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: **09-Feb-2018 – 08-Feb-2019**

Please remember to use your **Project ID [0751]** on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see *Forms and Instructions* on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

The HREC will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Departement of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website https://applyethics.sun.ac.za/ProjectView/Index/751

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

[redacted]

HREC Coordinator,

Health Research Ethics Committee 2 (HREC2).

National Health Research Ethics Council (NHREC) Registration Number:

REC-130408-012 (HREC1)·REC-230208-010 (HREC2)

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:
IRB0005240 (HREC1)·IRB0005239 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the World Medical Association (2013). Declaration of Helsinki:

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7. Appendix G: ART Procedures - Wijnland Fertility Clinic SOP's

WIJNLAND
Fertility

STANDARD OPERATION PROCEDURES

ASSISTED REPRODUCTIVE TREATMENT

Table of Contents

WIJNLAND
Fertility

STANDARD OPERATION PROCEDURES

ASSISTED REPRODUCTIVE TREATMENT

I. Ovarian stimulation

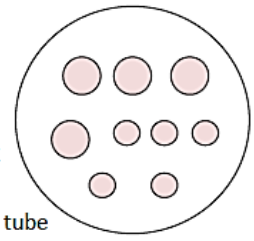
1. **Follicular stimulation:**
   a. Day 3 of menstrual cycle until trigger day with either HMG or Recombinant FSH
   b. LH suppresion with recombinant gonadotropin-releasing hormone (GnRH) antagonist from either day 8 of menstrual cycle or leading follicle of 14mm, which ever comes first, until trigger day
   c. Ovulation trigger when leading follicle ≥18mm with Recombinant HCG
2. **Estrogen supplementation:**
   a. Oral estrogen of 2mg per day according to prescription
3. **Luteal phase support:**
   a. Vaginal progesterone suppositories

II. Oocyte Retrieval

Acronyms:

- OPU = oocyte pick up
- COC = cumulus-oocyte-complex
- GT = Life Global Total medium range (IVF Online™)
- QA = Quinns Advantage culture medium range (Sage™)
- 1S = 1-Step with SPS or HSA culture medium (Sage™)

1. **Preparation procedure**
   a. Fertilization petri dish:
      i. Appropriate Fertilization medium in drops with tissue culture oil overlay
      ii. Label with patient identifier
      iii. Incubate ≥ 4 hours
   b. Start patient and embryo documentation
2. **OPU**
   a. Prepare test tube with buffered culture medium and warm to 37°C
   b. Recieve follicular fluid from aspiration done in theatre
   c. Identify COCs and aspirate with pipette into warm culture medium tube
   d. Place all COCs in fertilization dish after completion of COCs OPU



WIJNLAND
Fertility

STANDARD OPERATION PROCEDURES

ASSISTED REPRODUCTIVE TREATMENT

III. COC stripping for sperm injection

1. **Preparation procedure**
   a. Strip petri dish:
      i. Appropriate drops of hyaluronidase supplemented buffered culture medium
      ii. Appropriate buffered culture drops with oil overlay
      iii. Warm to 37°C
      iv. Label with patient identifier
2. **Stripping**
   a. Use appropriate stripping pipette or micropipettor
   b. Count number of initial COCs to correspond to final oocyte count
   c. Check oocyte maturity
   d. Transfer oocytes to fertilization dish into unused drops and replace to incubator
   e. Note maturity on patient documentation

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IV. Sperm preparation

1. **Preparation procedure**
   a. Processing tubes
      i. Aliquot appropriate amount of selected gradient(s) solution(s) in tube
      ii. Aliquot appropriate sperm wash/fertilization medium in tube
      iii. Warm to 35°C
      iv. Label both tubes with patient identifier
2. **Sperm processing**
   a. Fresh semen sample
      i. Allow complete liquefaction
      ii. Warm to 35°C
      iii. Evaluate on wet preparation slide of semen and document semen parameters
      iv. Aliquot appropriate amount of semen onto prepared gradient solution
   b. Frozen semen/biopsy tissue sample
      i. Retrieve appropriate patient sample straws from dewar
      ii. Allow complete thawing of straw(s)
      iii. Warm to 35°C
      iv. Evaluate and note sperm parameters on wet preparation slide
      v. Aliquot whole thawed sample onto prepared gradient/wash solution
   c. Centrifugation and wash
      i. Centrifuge at 300-450g for 10-25min
      ii. Discard supernatant
      iii. Place sperm pellet into prepared sperm wash/fertilization medium tube
      iv. Centrifuge at 300-450g for 10min
      v. Discard supernatant
      vi. Resuspend sperm pellet
      vii. Evaluate on wet preparation slide and document semen parameters
   d. Insemination
      a. IVF
         i. Place prepared sperm sample in incubator for equilibration
      b. Sperm injection
         ii. Place prepared sperm sample on bench until injection procedure

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V. Standard IVF Insemination

1. **Preparation procedure**
   a. Retrieve fertilization dish with patient COCs from incubator
      i. Check patient identifier – double witness
   b. Retrieve prepared sperm sample tube with sperm sample from incubator
      ii. Check patient identifier – double witness
2. **Sperm insemination**
   a. Retrieve sperm with pipettor
      i. Exact concentration of sperm to be used may be calculated
   b. Release sperm into drops with COCs
      i. Work under microscope
   c. Replace dish into incubator
   d. Document on patient form
3. **Inseminated COC stripping**
   a. Retrieve fertilization dish from incubator
   b. Identify COCs
   c. Retrieve COCs with pipettor
   d. Use appropriate stripping pipette or micropipettor
   e. Count number of initial COCs to correspond to final oocyte count
   f. Check oocyte maturity
   g. Transfer oocytes to fertilization dish into unused drops and replace to incubator
   h. Note maturity on patient documentation

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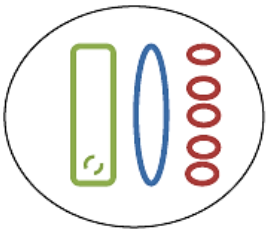
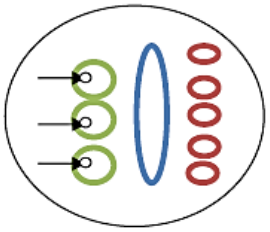
VI. Intra Cytoplasmic Sperm Injection (ICSI) and variations (PICSI, IMSI)





1. **Preparation procedure**
   a. ICSI/PICSI/IMSI dish:
      i. Appropriate drop(s) of buffered culture medium for gametes,
      ii. Appropriate drop(s) of PVP supplemented medium for manipulation
      iii. Cover with oil overlay
      iv. Label with patient identifier
   b. Manipulator:
      i. Load and set micropipettes for holding and injection
      ii. Warm heated stage to 37°C
      iii. Set up light configurations of microscope
2. **Sperm injection**
   a. Retrieve prepared sperm sample tube with sperm sample from bench
      i. Check patient identifier – double witness
      ii. Load sufficient sperm into allocated sperm drop in ICSI dish with pipet
   b. Retrieve fertilization dish with patient oocytes from incubator
      i. Check patient identifier – double witness
   c. Select individual sperm for injection of individual oocytes
   d. Load oocytes according to selected sperm
      a. Inject all oocytes with single selected sperm
      b. Replace injected oocytes into fertilization dish into new drop
   e. Document injection details onto patient form

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VII. Standard Embryo Culture

1. **Preparation procedure**
   a. Embryo culture dish:
      i. Appropriate drops of embryo culture medium for oocyte wash and culture
      ii. Cover with oil overlay
      iii. Label with patient identifier
2. **Inseminated oocyte loading**
   a. Retrieve fertilization dish with patient oocytes from incubator
      i. Check patient identifier – double witness
   b. Wash oocytes in embryo culture wash drops
   c. Allocate single oocytes to embryo culture drops
   d. Replace patient embryo culture dish to incubator
3. **Daily embryo culture and grading**
   a. Retrieve embryo culture dish from incubator
   b. Place embryo culture dish on heated ICSI manipulator stage
   c. Evaluate embryo development according to Istanbul Consensus embryo scoring method *(see References)*
   d. Replace patient embryo culture dish to incubator
   e. Document embryo evaluation on patient form
   f. Complete daily from oocyte to blastocyst stage until final status has been decided
      i. Allocate and document viable embryos for transfer, cryopreservation and non-viable embryos for disposal
   g. Remove embryo culture dish from incubator after completion of embryo culture and allocations

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STANDARD OPERATION PROCEDURES

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VIII. Time-lapse Embryo Culture

1. **Preparation procedure**
   a. EmbryoSlide® dish:
      i. Use appropriate embryo culture medium and pipettor
      ii. Prepare appropriate number of slide(s) according to number of oocytes retrieved
      iii. Prepare slides according to Vitrolife™ EmbryoSlide® preparation Technote (see Appendix A)
      iv. Cover with oil overlay
      v. Label with patient identifier
      vi. Equilibrate in incubator for ≥4 hours
   b. Initiate patient file on EmbryoViewer® station with patient details
2. **Inseminated oocyte loading**
   a. Retrieve EmbryoSlide® dish from incubator
      i. Check patient identifier – double witness
      ii. Remove bubbles from wells
   b. Retrieve fertilization dish with patient oocytes from incubator
      iii. Check patient identifier – double witness
   c. Wash oocytes in embryo culture wash wells
   d. Allocate all oocytes to single embryo culture wells
   e. Load EmbryoSlide® into EmbryoScope® according to manufacturer instruction manual
   f. Allocate EmbryoSlide® to patient
3. **Embryo annotation and grading**
   a. Annotate embryos according to Vitrolife™ EmbryoScope® embryo annotation Technote
   b. Complete daily from oocyte to blastocyst stage until final status has been decided
      i. Allocate and document viable embryos for transfer, cryopreservation and non-viable embryos for disposal
   c. Remove and end EmbryoSlide® from EmbryoScope® after completion of embryo culture and allocations

ASSISTED REPRODUCTIVE TREATMENT

IX. Embryo transfer

1. **Preparation procedure**
   a. Embryo transfer dish:
      i. Appropriate embryo culture medium for transfer with pipettor
      ii. Appropriate embryo culture medium for wash
      iii. Label with patient identifier
      iv. Equilibrate in incubator for ≥2 hours
   b. Identify, select and allocate embryo for transfer
   c. Retrieve embryo culture dish/EmbryoSlide® from incubator
      i. Check patient identifier – double witness
   d. Retrieve embryo transfer dish from incubator
      i. Check patient identifier – double witness
   e. Transfer selected embryo with pipet to transfer well/drop in prepared transfer dish
2. **Transfer procedure**
   a. Retrieve prepared embryo transfer dish with selected embryo loaded
      i. Check patient identifier – double witness
   b. Rinse syringe with embryo culture wash medium
   c. Connect transfer catheter with transfer syringe
   d. Aspirate embryo from embryo transfer medium into transfer catheter
   e. Hand loaded embryo transfer catheter to clinician for embryo transfer procedure
   f. Retrieve emptied embryo transfer catheter from clinician
   g. Place embryo catheter tip into empty well and disconnect syringe from catheter
      a. Check released embryo transfer medium for embryo retention
   h. Reload embryo in case of retention adn repeat transfer procedure

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X. Oocyte & Embryo Cryopreservation

1. **Preparation procedure**
   a. Vitrification dish:
      i. Wait for vitrification medium to reach room/ambient temperature
      ii. Prepare Repro Plate/Vitri Plate with appropriate vitrification mediums according to Kitazato/Cryotec instruction leaflet according to sample type *(See Appendices)*
      iii. Use pipettor
      iv. Label with patient identifier
   b. Storage device:
      i. Select appropriate Cryotop®/Cryotec®storage coloured device
      ii. Label with patient and straw identifiers
      iii. Check patient identifier – double witness
   c. Storage dewar:
      i. Find open storage goblet and allocate to patient samples
2. **Sample loading and vitrification procedure**
   a. Retrieve fertilization dish/embryo culture dish/EmbryoSlide® from incubator
      i. Check patient identifier – double witness
      ii. Identify selected patient oocyte/embryo for vitrification
   b. Retrieve oocyte/embryo from culture drop/well with pipet
   c. Load into vitrification medium according to Kitazato/Cryotec instruction leaflet *(See Appendices)*
   d. Follow vitrification procedures according to Kitazato/Cryotec instruction leaflet *(See Appendices)*
3. **Storage procedure**
   a. Retrieve allocated goblet from storage dewar
   b. Place into liquid nitrogen container with patient vitrification device loaded with samples
      i. Check patient identifier – double witness
   c. Place storage devices into goblet under level of liquid nitrogen
      i. Load all devices until goblet is full
   d. Retrieve filled goblet from liquid nitrogen and replace to original space in dewar
      i. Check patient identifier – double witness
   e. Document storage details on patient form

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STANDARD OPERATION PROCEDURES

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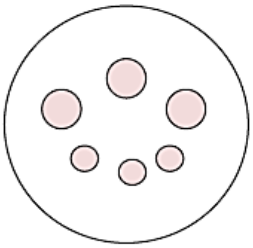
XI. Oocyte/Embryo thawing

1. **Preparation procedure**
   a. Fertilization/Embryo culture petri dish:
      i. Appropriate fertilization/embryo culture medium in drops
      ii. Cover with tissue culture oil overlay
      iii. Label with patient identifier
      iv. Equilibrate for ≥4hours
   b. Document on patient form
   c. Prepare thawing medium according to Kitazato®/Cryotech® product insert



   d. Thawing dish:
      i. Wait for thawing medium to reach room/ambient temperature
      ii. Prepare Repro Plate/Thaw Plate with appropriate thawing mediums according to Kitazato®/Cryotec® instruction leaflet according to sample type *(See Appendices)*
      iii. Use pipettor
      iv. Label with patient identifier
   e. Storage dewar:
      i. Identify allocated storage goblet with patient samples
      ii. Check patient identifier – double witness
      iii. Retrieve goblet from dewar and place directly under liquid nitrogen level
   f. Storage device:
      i. Identify and retrieve storage device with selected patient sample in liquid nitrogen
      ii. Check patient identifier – double witness
      iii. Replace goblet to original space in dewar, if applicable, with remaining patient storage devices
2. **Thawing**
   a. Thaw sample according to Kitazato®/Cryotech® product insert
   b. Note survival on patient documentation

A. Vitrolife™ instructions for EmbryoSlide® preparation for embryo culture in the EmbryoScope®

TechNOTE Vitrolife

Preparation of EmbryoSlide Culture Dishes

The EmbryoSlide® culture dish is specifically designed for the individual culture of up to 12 embryos in the EmbryoScope™ time-lapse incubator. The dish also contains wells designed for rinsing.
The EmbryoSlide culture dishes are designed for easy and stable handling, and are made of culture-tested polystyrene. They are delivered in sterile, single pouches.

Vitrolife recommends preparation of EmbryoSlide culture dishes on the day before use. Prepare the dishes with cold medium and on a non-heated surface to avoid evaporation. The procedure described below requires less than 1.5 minutes per dish.

General characteristics of the EmbryoSlide culture dish

The embryos are incubated in individual microwells in a small (25 µl) volume of culture medium under a confluent oil cover.

Each well carries a number from 1-12 for identification under a stereo microscope. Each well number corresponds to the well identification number in the EmbryoViewer® software.

Two rinsing wells are available at each end of the dish. These special wells can be used during embryo handling (identified as A-D).

There is a slight variation in how much the temperature decreases in the microwells (approx. 0.6°C) and the rinsing wells (approx. 0.7°C). Both measurements have been performed on a 37°C heating plate over a period of two minutes. This represents normal dish handling time.

Each batch of EmbryoSlide+ culture dishes must pass our stringent MEA testing procedure before being released for sale. This is part of the Vitrolife quality assurance.

Preparation for use on the next day

Prepare the EmbryoSlide culture dishes on the day before use. Prepare one dish at a time to minimise the handling time of each dish.

The EmbryoSlide culture dishes should be prepared with cold medium and oil on a non-heated workbench to avoid evaporation of the medium during preparation.

When they have been prepared, the culture dishes must equilibrate overnight before loading embryos into the microwells.

Use a stereo microscope to control the process.

The recommended procedure for preparing the culture dishes is outlined on the next page.

TECHNOTE: Preparation of EmbryoSlide culture dishes, Vitrolife, v.11 INT, JUNE 2018

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Step	Action
	**Remove the culture dish from the pouch.** **Prepare the dishes with cold culture medium and oil on a non-heated workbench to avoid evaporation.** **Prepare one dish at a time to minimise the handling time of each dish.**
	**Fill all microwells with a small amount of culture medium*** **Use a micropipette.** Slightly overfill the microwell to create a convex meniscus.
	**Immediately fill all needed wells, including the rinsing wells, with 25 µL of culture medium*. Use a standard pipette.**
	**Immediately load 1.4 mL of culture oil* into the reservoir** It is important to apply the oil overlay quickly to avoid evaporation of medium. Make sure that all wells, including the rinsing wells, are covered with a confluent oil layer to eliminate evaporation of medium. Add an additional 25µL of culture oil per well not filled with medium.
	**Push up larger bubbles with a micropipette and remove them** **Cover with the lid and equilibrate overnight.** **Remove any bubbles that may have formed.** **Load embryos into the center of microwells. Use a micropipette.**
	**Place the dish in the EmbryoScope incubator.**

*Vitrolife recommends using G-TL medium, designed specifically for continuous culture with time-lapse technology and OVOIL™ 100% paraffin culture oil for complete control of your culture system. Vitrolife products are produced under highly controlled processes.

Refer to the TECHNOTE *Additional notes on EmbryoSlide® culture dish preparation* for further recommendations about optimal handling of the EmbryoSlide culture dish.

TECHNOTE: Preparation of EmbryoSlide culture dishes, Vitrolife, v.11 INT, JUNE 2018

TECHNOTE

Vitrolife

Additional notes for EmbryoSlide® culture dish preparation

This TECHNOTE describes additional procedures and information related to the handling and preparation of EmbryoSlide® culture dishes.

The handling of EmbryoSlide culture dishes is described in the TECHNOTE "Preparation of EmbryoSlide® culture dishes".

EmbryoSlide culture dishes: preparation for use on the same day

Although preparation of EmbryoSlide culture dishes is recommended one day before use, there may be circumstances requiring preparation of a culture dish for use on the same day.

The procedure follows essentially the one described in the TECHNOTE "Preparation of EmbryoSlide® culture dishes" except that the use of pre-warmed and pre-gassed/equilibrated medium is mandatory.

Culture dishes prepared with pre-gassed/equilibrated medium should be re-equilibrated after preparation for another 2-4 hours before embryos are loaded in the micro-wells. This serves mainly to stabilize the temperature.

Removal of occasional air bubbles

Usually the above method of filling does not produce air bubbles but all wells need to be carefully checked.

If air bubbles are present after preparation remove all bubbles in the well and in the oil layer immediately. However, small bubbles and bubbles in the micro-well can be more easily removed after equilibration.

- If air bubbles are present at the interface between the medium and the oil they should be removed immediately with a standard pipette containing media.

  By capillary effect the bubbles will aspirate into the pipette tip when this is placed close to the air bubble

- If air bubbles are present at the bottom of the micro-well or small bubbles are sticking to the side of the well it is recommended to incubate the EmbryoSlide culture dish in an incubator for 1-2 hours as this will cause the bubbles to grow and to round up for easier removal.

  Once the bubbles have rounded up simply touching them with a micro pipette tip will cause them to swim up and they can be easily removed without dragging oil into the micro-well.

The EmbryoSlide® culture dish

TECHNOTE: Additional notes for EmbryoSlide® culture dish preparation, Vitrolife, v.3 INT, AUGUST 2015

ASSISTED REPRODUCTIVE TREATMENT

B. Vitrolife™ instructions for embryo annotations for embryo grading in the EmbryoScope®

TechNOTE

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Consistent annotation for better evaluation – a guide on definitions for morphokinetics

Annotations constitute the base on which embryo evaluation can be performed using time-lapse monitoring in the IVF clinic.
The embryo developmental events that can be detected with time-lapse technology are immense. Events relevant for annotation ideally reflect embryonic potential in the specific clinical setting. Therefore it is important to define which events are relevant for the evaluation of embryos in your clinical setting.

Annotations should be objective and definitions should be the same across operators in order to perform meaningful evaluations.
This technote describes definitions of variables most commonly used in embryo assessment with time-lapse. These definitions will help you attain consistent annotations and thereby objective evaluations in your clinic and further streamline the understanding of embryo developmental events within the clinic and beyond the clinic.

Evaluation of embryos with KIDScore models require only few annotations, however this technote describes a more extensive selection of variables.

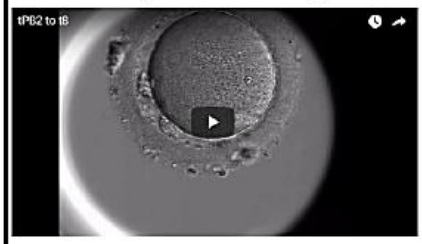
Time-lapse assessment

The first step on the way to reach consistency of annotations within a clinic is to agree on definitions of each annotated variable.
Time-lapse facilitates a more precise and objective method of embryo assessment than with static embryo monitoring. This is due to the continuous monitoring provided by time-lapse technology.

This continuous monitoring allows you to visually detect changes in embryo stages and morphology in a precise manner.

Morphokinetics – assessing embryo stages

With time-lapse the exact time that an embryo transits into a new stage can be determined with precision.
To do this, visual differences from one image to the next should be registered as annotations.
With morphokinetic variables, annotating the first time that an embryo is observed to be in a certain stage ensures a consistent and objective annotation strategy.



Annotation of fertilization events and blastomere cleavages: tB2, tPNa, tPNf, t2, t3, t4, t5, t6, t7 and t8

Variables from tPB2 to t8 represent distinct events that are detectable by differences from one image to the next.
To annotate those, the first image for which the stage is observed is annotated.
tPB2; time of extrusion of 2nd Polar Body: annotate at the first image in which the 2nd polar body is observed.
tPNa; time of ProNuclei appearance: annotate at the first image in which all pronuclei can be observed.
tPNf; time of ProNucei fading: annotate at the first image in which all pronuclei have faded.
t2-t8; time of cleavage to 2 etc cells: annotate at the first image in which a distinct separation of cell membranes can be observed, i.e. mark the exact time that the embryo progresses into another developmental stage.

The video to the left illustrates the definitions of morphokinetic variables from tPB2 to t8. View the full video at www.vitrolife.com

TECHNOTE: Making consistent annotations for better evaluation, Vitrolife A/S, v.2, NOVEMBER 2017

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Annotation of morula and blastocyst formation

Morula and blastocyst formation are both processes that are not observed as instantaneous occurrences but rather observed as reached through gradual, subtle changes.

In order to reach consistency when annotating developmental steps during morulation and blastulation, definitions are based on distinctive features during the gradual processes.

time of Starting Compaction (tSC): the first time that membranes between some of the blastomeres of the future morula are no longer distinct.

time of Morula (tM): the first image in which a compacted morula includes all the blastomeres that will later take part in the formation of the blastocyst. This solves the question of how to handle partial compactions as excluded cells can be accounted for

time of Starting Blastulation (tSB): the first time that a sign of cavity formation is observed. As the blastocoel cavity grows during blastulation, going back in the image sequence from a definite blastocyst stage can be helpful to attain this annotation.

time of Full Blastocyst (tB): the last image before expansion starts. This is recognised as the last image before the zona pellucida is pushed by the growing blastocyst. This is a very distinct hallmark during blastocyst development and therefore easy to annotate precisely and consistently.

time of Expanding Blastocyst (tEB): blastocyst expansion can go on for several hours and therefore a defined characteristic during this process is necessary to obtain accuracy during embryo analysis. Importantly, this should be informative on another level than previous parameters as otherwise annotation would be dispensable. Therefore, we characterize tEB as the time at which the blastocyst has expanded so much that the zona pellucida has reached half of its original thickness, which can be measured and thus represents a truly objective assessment.

time of Hatching Blastocyst (tHB): the first image at which a sign of hatching is observed.

View the full videos at www.vitrolife.com

Note that for some variables precise and consistent annotation is easier if the video sequence is followed backwards in time, i.e. from a time of definite observation to the exact time of first observation.
This is especially helpful for variables which occur gradually and hence do not evoke extensive changes between consecutive images such as e.g. time of ProNuclear appearance (tPNa) and time of Starting Blastulation (tSB).
The above definitions reflect time-lapse annotations as recommended by Vitrolife and to some extend based on the definitions of Ciray et al., 2014: Hum Reprod 29(12): 2650-2660

TECHNOTE: Making consistent annotations for better evaluation, Vitrolife A/S, v.2, NOVEMBER 2017

ASSISTED REPRODUCTIVE TREATMENT

C. Istanbul consensus for embryo grading during standard embryo culture

Standardized Grading Sheme for Morphological Assessment of Embryos

Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting☆

Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology [1,*]

Reproductive BioMedicine Online (2011) 22, 632–646

i. Istanbul consensus for fertilization check during standard embryo culture

Fertilization check

The optimal fertilized oocyte should be spherical, and have two polar bodies, with two centrally located, juxtaposed pronuclei that are even sized, with distinct membranes. The pronuclei should have equivalent numbers and size of NPBs that are ideally equatorially aligned at the region of membrane juxtaposition.

It was agreed that both pronuclear size and location should be assessed at fertilization check (Table IV). The consensus was that the following features of pronuclei are severely atypical: widely separated pronuclei; pronuclei of grossly different sizes; micronuclei. The presence of sER disks should be assessed as part of the fertilization check (if IVF, rather than ICSI was performed). Normally fertilized oocytes in which sER disks are observed should not be transferred.

The consensus was that at present, there is insufficient evidence to support a prognostic value for the observation of a peripheral cytoplasmic translucency in the fertilized oocyte (a 'halo').

The decision to perform a second Day 1 assessment is at the discretion of the laboratory, and may be either a syngamy or an early cleavage assessment (Table IV). The purpose of the second assessment can be for either quality control (syngamy) or prognostic (early cleavage) reasons, which will define the assessment time selected.

ASSISTED REPRODUCTIVE TREATMENT

ii. Istanbul consensus for embryo grading at cleavage stage during standard embryo culture

Table VI

Consensus scoring system for cleavage-stage embryos (in addition to cell number).

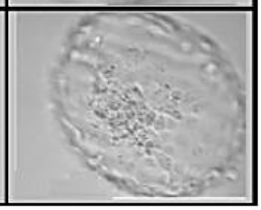
Grade	Rating	Description
1	Good	• <10% fragmentation
		• Stage-specific cell size
		• No multinucleation
2	Fair	• 10–25% fragmentation
		• Stage-specific cell size for majority of cells
		• No evidence of multinucleation
3	Poor	• Severe fragmentation (>25%)
		• Cell size not stage specific
		• Evidence of multinucleation

D. Modified Gardner blastocyst grading system for blastocyst expansion grading using a numerical scoring system suggested by the Istanbul consensus document

Blastocyst Grade	*Description criteria*
CM: Compact morula	Compacted morula
EB: Early:	Start of formation of blastocoel, no ICM and TE distinguishable
Grade 1: Cavitating:	the blastocoel cavity is <50% of the volume of the embryo, ICM and TE distinguishable
Grade 2: Full:	the blastocoel cavity is ≥60-100% of the volume of the embryo, until completely filling the PVS
Grade 3: Expanding:	starts to expand until ZP is 10-70% thinned
Grade 4: Expanded:	ZP is >70% thinned
Grade 5: Hatching:	ZP is breached, trophectoderm cells protrudes from ZP
Grade 6: Hatched:	More ≥50% hatched out of the ZP

ASSISTED REPRODUCTIVE TREATMENT

E. Gardner blastocyst grading system for blastocyst Trophectoderm (TE) and Innercell mass (ICM) during embryo culture

Inner Cell Mass (ICM)	***A*** *Numerous and tightly packed*	***B*** *Several and loosely packed cells*	***C*** *Few cells*
Trophectoderm (TE)	***A*** *Many tightly packed cells organised into epithelium*	***B*** *Several cells organised into loose epithelium*	***C*** *Few cells*
Morula			
Early Blastocyst			
Blastocyst			
Expanded Blastocyst			
Hatching Blastocyst			
Fully Hatched Blastocyst			

ASSISTED REPRODUCTIVE TREATMENT

F. Vitrolife™ instructions for blastocyst grading annotations in the EmbryoScope®

TECHNOTE Vitrolife

Guidelines for blastocyst morphology grading with time-lapse

Grading blastocyst morphology with the use of time-lapse technology facilitates a more thorough evaluation because the complete course of development can be considered. This means that e.g. cells that are excluded during compaction or subsequent blastocyst formation can be accounted for. Similarly, fragments disturbing the visual impression of a blastocyst can be identified as all focal planes can be reviewed throughout embryo development.

Altogether, this means that a comprehensive impression of the blastocyst can be used as the basis for grading morphology. This should be utilized when grading ICM and TE and is necessary when using KIDScore D5.

Blastocyst grading for KIDScore D5

Time-lapse monitoring of embryos gives you a different level of information regarding development of both ICM and TE. This includes number of cells that each layer originates from and extrusion of cells during the compaction or expansion phase. This information must be taken into account when grading blastocysts for KIDScore D5 application.

To use KIDScore D5 a separate and independent grade from A to C must be given for both ICM and TE for each embryo reaching the blastocyst stage. Grade "A" defines cell layers with highest quality morphology whereas grade "C" defines cell layers with lowest quality morphology.

Definitions for each grade for both ICM and TE is defined below.

ICM grade	Description
A	Many tightly packed cells. Cell boundaries are not distinct and the layer is homogenous without vacuoles and debris.
B	Several cells and the layer can be less tightly packed. The layer can be less homogenous and few vacuoles or minor degenerations may be observed.
C	Very few cells that are loosely packed. Cells may be large and show distinct boundaries. The size of the ICM may differ in this group as a few big cells lead to an overall larger size. The larger size is, however, the result of poor compaction. The layer may show vacuoles, degenerated cells or independent cells. This grading group also covers cases where the ICM is not distinguishable.

TE grade	Description
A	Many flattened cells (often >40) forming a cohesive layer that lines the blastocoel cavity. The cells often contain clearly visible nuclei and the cytoplasm is homogenous.
B	Several (often > 20) cells. The layer is not completely cohesive and the shape of the cells varies within the layer. Cell cytoplasm may appear non-homogenous and it may be difficult to distinguish nuclei.
C	Very few cells which are often large and stretched over a large area. Cytoplasm often appears non-homogenous and vacuoles may be present.

For both layers the grade "N/A" is given to embryos in case the cell layer can not be evaluated.

TECHNOTE: Guidelines for blastocyst morphology grading with time-lapse, Vitrolife A/S, v.3, AUGUST 2016

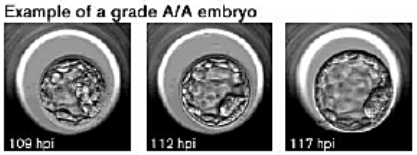
TECHNOTE

Vitrolife

Examples of blastocyst morphology grades with time-lapse

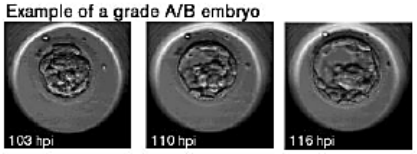
Below you can see the progression of some examples of blastocyst development with associated ICM/TE grades. A short description to illustrate the grade is given next to each embryo. Time is given in hours post insemination (hpi).

Example of a grade A/A embryo



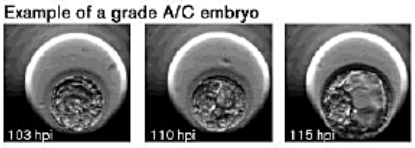
ICM is large, originates from many cells and ends up as a tightly compacted layer.
TE originates from many cells that end up forming a cohesive layer lining the blastocoel cavity.

Example of a grade A/B embryo



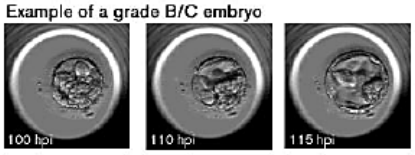
ICM is composed of many cells and is tightly compacted.
TE is composed of several cells but varies in size and cohesiveness.
Note: At 103 hpi the embryo shows a blastomere that is pushed into the perivitelline space and does not take part in blastocyst formation. At 116 hpi this blastomere is degenerated and appears as debris in the same position.

Example of a grade A/C embryo



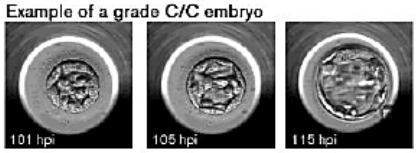
ICM is large and originates from many cells which are tightly compacted.
TE is composed of few, large cells and some are stretched over a long distance.

Example of a grade B/C embryo



ICM consists of several cells and is loosely compacted.
TE consists of very few and large cells.

Example of a grade C/C embryo



ICM is composed of few cells. Image 2 (105 hrs) shows a "bridge" that connects the two cell layers.
TE originates from few cells that are large and stretches over a long distance.
Note: The large cellular debris (fragmentation) pushed into the perivitelline space is not part of the actual blastocyst

For KIDScore D5 to work as intended, the guidelines described here should be followed and blastocyst morphology grades must be annotated between 115 and 120 hours after insemination.

TECHNOTE: Guidelines for blastocyst morphology grading with time-lapse, Vitrolife A/S, v.3, AUGUST 2016

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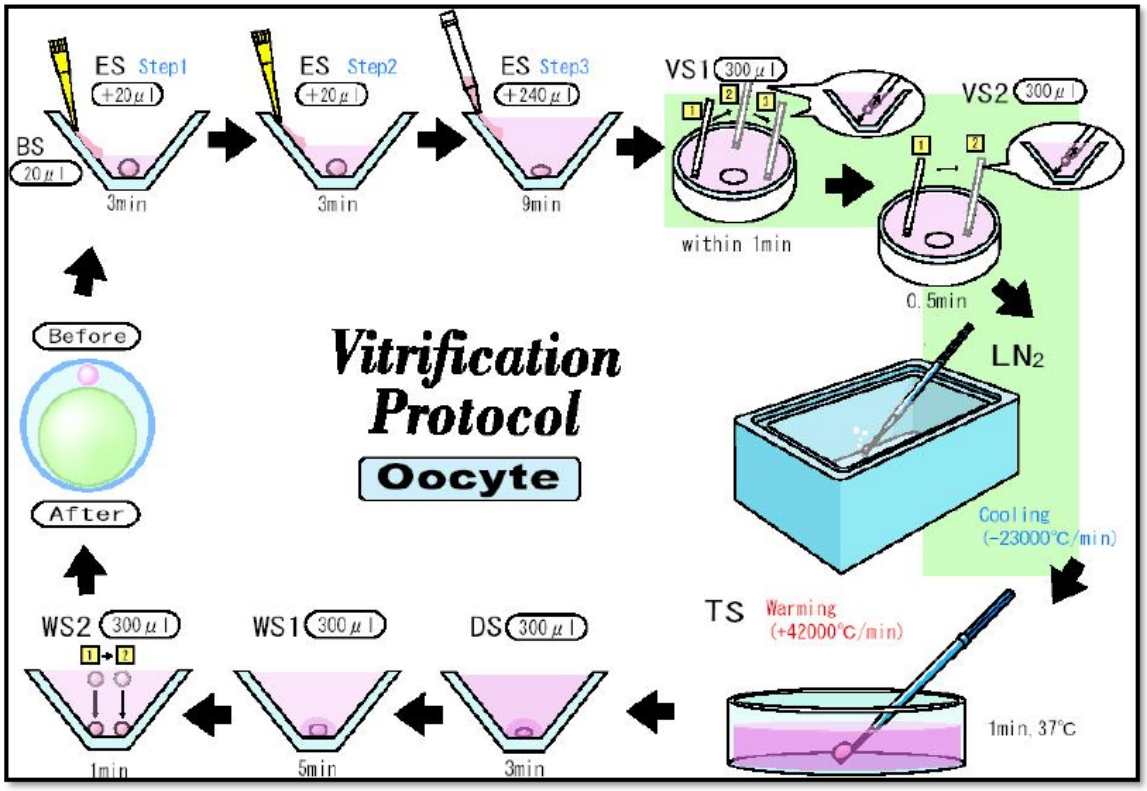
STANDARD OPERATION PROCEDURES

ASSISTED REPRODUCTIVE TREATMENT

G. Kitazato™ instructions for oocyte vitrification and thawing with the Cryotop® method



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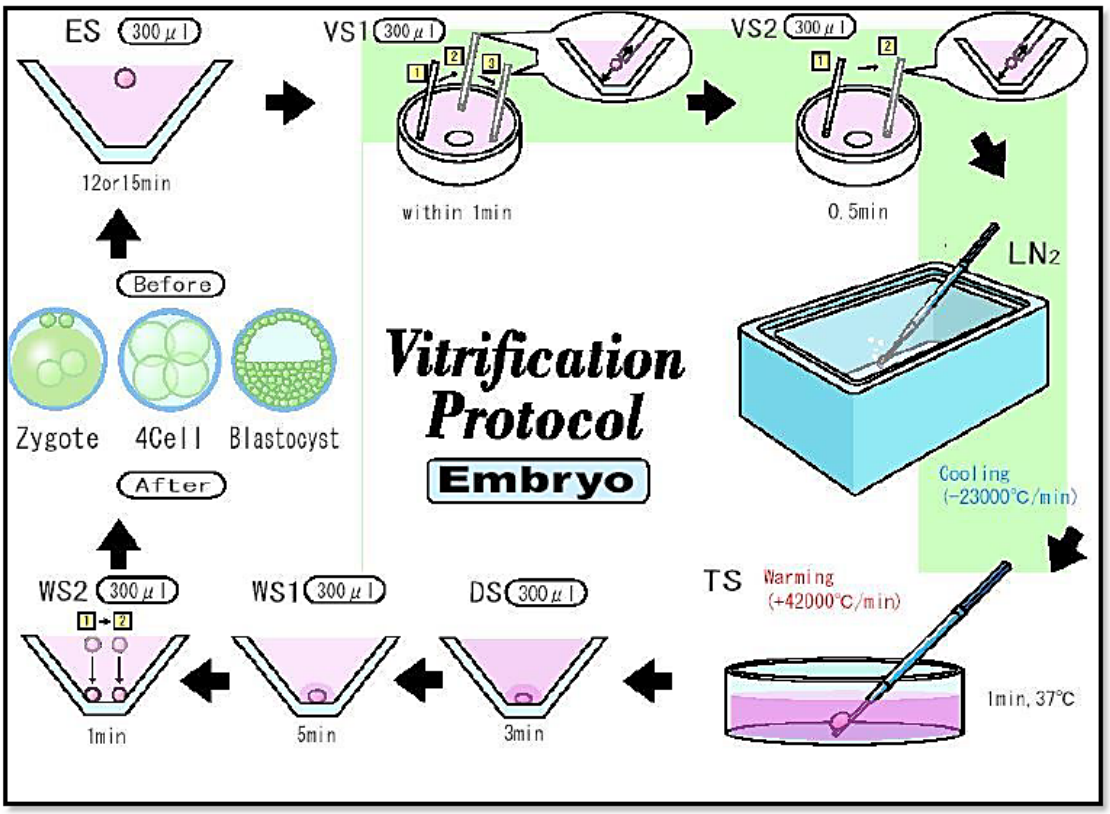
STANDARD OPERATION PROCEDURES

ASSISTED REPRODUCTIVE TREATMENT

H. Kitazato™ instructions for embryo vitrification and thawing with the Cryotop® method



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Fertility

STANDARD OPERATION PROCEDURES

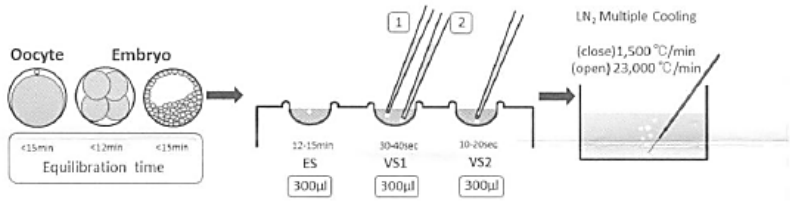
ASSISTED REPRODUCTIVE TREATMENT

I. Cryotech™ instructions for vitrification with the Cryotop® method

Cryotech®

For Oocytes and Embryos

VITRIFICATION SOLUTION SET (110) : For 10 times Uses



Contents:
-2 vials of 1.8 ml Equilibration Solution (ES).
-4 vials of 1.8 ml Vitrification Solution (VS).

Instructions:

Preparation
-The whole process should be performed under room temperature (25-27°C).
-Fill a nitrogen container.
-Compare the thickness of the zona pellucida with the perivitelline space, and take note for oocytes.
-Important : Use a right diameter size of Pasteur pipette for oocytes and embryos (140-150 µm), and blastocysts (160-200 µm).

Equilibration of oocytes and embryos

1. Fill in the 1st well of a Vitri Plate with 300 µl of ES, and 300 µl of VS in both of the 2nd and 3rd well.
2. Put an oocyte/embryo on the surface of ES in the 1st well.
3. The oocyte/embryo will sink and begin to shrink, and gradually return to the original size (maximum time is 15 min for oocytes and blastocysts, and 12 min for other stages of embryos).

Vitrification

Attention: The following steps must be taken in more than 25 sec and less than 90 sec.

4. Transfer the oocyte/embryo from the 1st well to the half depth of the 2nd well with VS. (Not with the minimum volume of ES at the first step) The oocyte/embryo immediately floats to the surface of VS while being washed.
5. After washing the inside wall of the pipette with fresh VS media, take only the oocyte/embryo and transfer it to the bottom of the well. Wait until the oocyte/embryo stops floating in VS.
6. Transfer the oocyte/embryo to the half depth of the 3rd well with VS, and mix the media by pipette around for 5 times.
7. Take only the oocyte/embryo at the tip of the pipette, and put it on the end of the Cryotec seat with a minimum volume of VS.
8. Immediately submerge the Cryotech into liquid nitrogen.
9. Place the cap, and store the Cryotec in a nitrogen tank.

Please leave the Cryotec in liquid nitrogen at all times.

Quality Control Tests:

-Solutions
Lot is labelled on the vial of themselves.

Successfully passed the following controls.
- Sterility : Sterility test .
- Endotoxin by KT methodology (Each component).
- Efficiency: survival of 50/50 mouse embryos and porcine oocytes.

Storage and stability:
Solutions and kits can be transported under the room temperature, and then must be kept in the fridge at 2-8°C until the expiration date.
Use before the expiration date indicated on the each of the labels.

Composition:

-Modified HEPES Buffered MEM
-Hydroxy Propyl Cellulose
-Ethylene Glycol
-Dimethyl Sulfoxide
-Endotoxin free Trehalose

References:

- Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The CryoTop method. Theriogenology 67, 73-80, 2007.
- Cobo A, Kuwayama M. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. Fertil Steril. J59(6): 1657-64, 2007.
- Antinori M, Licata E, Dani G, Cerusico F, Versaci C, Antinori S. Cryotop vitrification of human oocytes results in high survival rate and healthy deliveries. Reproductive BioMedicine Online 14, 5-667, 2007.
- Vajta G, Kuwayama M. Improving cryopreservation systems. Theriogenology 65(1), 236-44, 2006.
- Kuwayama M. Highly efficient vitrification method for cryopreservation of human oocytes. Reproductive BioMedicine Online 11:300-308, 2005.
- Ushijima J, Kuwayama M. High survival rate of bovine oocytes matured in vitro following vitrification. J Reprod Dev. 50:685-96, 2004.
- Fukui Y, Kuwayama M. Effect of cryodevice type and donor's sexual maturity on vitrification of minke whale oocytes at germinal vesicle stage. Zygote 12, 333-338, 2004.
- Hochi S, Kuwayama M. Improved Survival of Vitrified in vivo-derived porcine embryos. J. Reprod. Develop. 50, 481-486, 2004.
- Esaki R, Kuwayama M. Cryopreservation of porcine embryos derived from in vitro-matured oocytes. Biology of Reproduction. 71, 432-437, 2004.

Product only for in vitro use.

Ver. 2
Revised 1th AUG 2014

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STANDARD OPERATION PROCEDURES

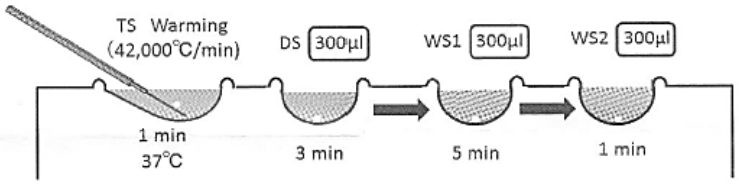
ASSISTED REPRODUCTIVE TREATMENT

J. Cryotech™ instructions for thawing with the Cryotop® method

Cryotech®

For Oocytes and Embryos

WARMING SOLUTION SET (205) : For 5 times Uses



Contents:
-5 vials of 1.8 ml Warming Solution (TS).
-1 vial of 1.8 ml Diluent Solution (DS).
-2 vials of 1.8 ml Washing Solution (WS).

Instructions:

Preparation
-The whole process should be made under room temperature (25-27°C).
-Important : Use a right diameter size of Pasteur pipette for oocytes and embryos (140-150 µm), and blastocysts (160-200 µm).

-Place a Warm plate and TS vial (with rid) in an incubator at 37°C 3 hours before the use (overnight storage is preferable).

-Expose DS and WS vials to the room temperature air at least 1 hour before the use.

-Take a patient's cane out of a liquid nitrogen tank, and take off the cover cap and prop up the Cryotec to the inside wall of the cooling rack with liquid nitrogen learning it against the wall.

Warming and dilution of CPAs

1. Take the Warm Plate out of the incubator and fill the second well with 300µl of DS.
2. Take the TS vial out of the incubator, and expel TS to the 1st square well.
3. Quickly (within 1 sec) put the Cryotec into the 1st square well with TS, and wait for 1 min.
4. Aspirate the oocyte/embryo and 3 mm long of TS into the pipette, and expel them most slowly to the bottom of the 2nd well (DS). Wait for 3 min.
5. While waiting, fill the 3rd(WS1) and 4th wells(WS2) with 300µl of WS each.
6. Aspirate the oocyte/embryo and 3 mm long of DS into the pipette, and expel them most slowly to the bottom of the 3rd well (WS1), and wait for 5 min.
7. Give a survival judgment at the end of this step if the shrunk oocyte/embryo is recovered or not.
8. Put the oocyte/embryo on the surface of the 4th well (WS2). When they sink and reach to the bottom, put them again on the surface of the same WS2 to wash for 2 times in total.
9. Put the oocyte/embryo in the droplet of the culture media for the recovery for ICSI and ET.

Note: 2 to 4 hours of culture for oocytes, and 3 hours for embryos.

Quality Control Tests:

-Solutions
Lot is labeled on the vial of itself.

Successfully passed the following controls.
- Sterility : Sterility test .
- Endotoxin by KT methodology (Each component).
- Efficiency: survival of 50/50 mouse embryos and porcine oocytes.

Storage and stability:

Solutions and kits can be transported under the room temperature, and then must be kept in the fridge at 2-8°C until the expiration date.
Use before the expiration date indicated on the each of the labels.

Composition:

-Modified HEPES Buffered MEM
-Hydroxy Propyl Cellulose
-Endotoxin free Trehalose

References:

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Revised 1th AUG 2014

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